S. Mohan Jain
P. M. Priyadarshan
EDITORS

Breeding Plantation Tree Crops

Tropical Species

Springer

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Preface

Tree species are indispensable to support human life. Due to their long life cycle and environmental sensitivity, breeding trees to suit day-to-day human needs is a formidable challenge. Whether they are edible or industrial crops, improving yield under optimal, sub-optimal and marginal areas calls for unified efforts from the scientists around the world. While the uniqueness of coconut as *kalpavriksha* (Sanskrit-meaning tree-of-life) marks its presence in every continent from Far East to South America, tree crops like cocoa, oil palm, rubber, apple, peach, grapes and walnut prove their environmental sensitivity towards tropical, sub-tropical and temperate climates. Desert climate is quintessential for date palm. Thus, from soft drinks to breweries to beverages to oil to tyres, the value addition offers a spectrum of products to human kind, enriched with nutritional, environmental, financial, social and trade related attributes.

Taxonomically, tree crops do not confine to a few families, but spread across a section of genera, an attribute so unique that contributes immensely to genetic biodiversity even while cultivated at the commercial scale. Many of these species influence other flora to nurture in their vicinity, thus ensuring their integrity in preserving the genetic biodiversity. While wheat, rice, maize, barley, soybean, cassava and banana makeup the major food staples, many fruit tree species contribute greatly to nutritional enrichment in human diet. The edible part of these species is the source of several nutrients that makes additives for the daily diet of humans, for example, vitamins, sugars, aromas and flavour compounds, and raw material for food processing industries. Tree crops face an array of agronomic and horticultural problems in propagation, yield, appearance, quality, diseases and pest control, abiotic stresses and poor shelf-life.

Shrinkage of cultivable land and growing demand has enforced these crops to be grown under marginal conditions that call for concerted efforts of plant breeders to go for the genetic improvement of these crops. A lot of research has already been done and is continued to preserve and utilise germplasm for genetic improvement of fruit crops, consumed for nutrition and commercial uses, for growing under environmental stress constraints. The published results are mainly available in the refereed journals and popular magazines. The researchers and scientists have to spend precious time in digging out the desired research references. The compilation of scientific data in the form of a book would certainly help a great deal in

providing information to the scientific community and industry people. There are few books available, which lack recent comprehensive information on a package of conventional breeding, biotechnology and molecular tools in crop improvement. With the use of modern molecular and biotechnological tools, the task of improving yield in tree crops is foremost in the acumen of future global agricultural research for sustainable production. This 2-volume book series deals with both tropical and temperate species, and is a sincere effort towards compiling the available research worldwide and bring them to the reference of scientists, researchers, teachers, students, policy makers and even planters. It is worthwhile to note that in the forthcoming years, tree crops are to be given much importance on par with annual crops due to carbon trading and nutritional up-gradation of the daily diet.

This book volume on tropical species deals with a total of 16 chapters on fruits and nuts (banana, mango, guava, papaya, grape, date palm, litchi, avocado and cashew), oil crops (coconut, oil palm and olive), industrial crops (rubber) and beverages (coffee, tea and cocoa). The second volume will deal with mainly temperate species.

The invited contributory authors are internationally well known specialists in individual crops. We highly appreciate their untiring efforts rendered in ensuring the inclusion of latest research accomplishments and their co-operation in revising their manuscripts timely. A few reviewers spared their valuable time in improving the quality of manuscripts. We are immensely thankful to them for their valuable help. Finally, we thank SPRINGER for bringing out this series to the readers.

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Part I
Fruit and Nut Crops

Chapter 1
Genetic Improvement of Banana

Frédéric Bakry, Françoise Carreel, Christophe Jenny, and Jean-Pierre Horry

1.1 Introduction

World production of bananas, estimated at 106 million tons (Lescot 2006), ranks fourth in agricultural production. Bananas make up the largest production of fruits and the largest international trade, more than apple, orange, grape and melon. Bananas are cultivated in more than 120 countries in tropical and subtropical zones on 5 continents. Banana products represent an essential food resource and have an important socioeconomic and ecological role.

Current varieties are generally seedless triploid clones either of the single genome A from the species *Musa acuminata* (group AAA) or of both genomes A and B from species *M. acuminata* and *Musa balbisiana* (groups AAB and ABB). More rarely, diploid varieties (AA and AB) and tetraploid clones are encountered. There are two major channels of banana production: those cultivated for export and those reserved for local markets. The main banana varieties cultivated for export, known as 'Grande Naine', 'Poyo' and 'Williams', belong to the monospecific triploid bananas (AAA) of the Cavendish sub-group. They differ from each other only in somatic mutations such as plant height or bunch and fruit shape. Their production relies on an intensive monoculture of the agro-industrial type, without rotation, and a high quantity of inputs.

Banana cultivation for local consumption is based on a large number of varieties adapted to different conditions of production as well as the varied uses and tastes of consumers. Diploid bananas, close to the ancestral wild forms, are still cultivated in Southeast Asia. In other regions, triploid clones belonging to different sub-groups – Plantain, Silk, Lujugira, Gros Michel, Pisang Awak – are the most widely distributed.

Bananas have many uses. They are not only consumed as fresh fruits but also cooked, like plantains. They are processed in various ways, into chips, fries, fritters,

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purees, jams, ketchup and alcohol (banana wine and beer have a very significant production in East Africa). The daily per capita consumption of bananas from 30 g to over 500 g in some East African countries. Apart from the fruit, other parts of the plant are also used: the pseudostem is used for its fibres and as floaters (*Musa textilis* or abacá) in the Philippines, and the leaves are used to make shelters or roofs or as wraps for cooking. In Thailand, the floral buds of particular varieties (Pisang Awak) are used in various culinary preparations. Some varieties are also considered to have medicinal properties.

Cultivated throughout the world, bananas are threatened by several diseases and pests (Stover and Simmonds 1987; Jones 1999) that need to be taken into account for banana improvement. Various major fungal diseases are constraints in industrial production and, to a lesser degree, in local production. For example, Sigatoka disease (SD) due to *Mycosphaerella musicola* and black leaf streak disease (BLSD) caused by *M. fijiensis* result in production losses in large industrial plantations and necessitate costly pest control measures to be adopted. In certain production zones, *Fusarium* wilt due to the soil fungus *Fusarium oxysporum* f. sp. *cubense* prevents the cultivation of susceptible varieties like the Gros Michel types. Great constraints are also exerted by the nematodes – *Radopholus similis* and several representatives of the genus *pratylenchus* – and by the black weevil of banana, *Cosmopolites sordidus*. Also, viral diseases are spreading. Those of greatest concern are due to BBTV (banana bunchy top virus), CMV (cucumber mosaic virus), BSV (banana streak virus) and BBMV (banana bract mosaic virus).

Chemical control measures used in intensive cultivation are not available to small banana farmers in developing countries. Furthermore, for some diseases, there is no effective chemical control. Genetic improvement has thus been focused mainly on obtaining varieties resistant to principal pests and diseases. Breeding bananas through hybridisation, which began in the 1920s, is currently being pursued at seven research centres. FHIA in Honduras is breeding banana for export as well as the 'cooking' types (Rowe 1984). EMBRAPA-CNPMF in Brazil (Dantas et al. 1993), NRCB and TNAU in India (Sathiamoorthy et al. 2000; Krishnamoorthy and Kumar 2004) aim at breeding local types of dessert and cooking bananas. CARBAP (Jenny et al. 2003) in Cameroon and IITA (Tenkouano and Swennen 2004) in Nigeria are conducting research on plantain and banana breeding in Africa. These six research centres are mainly interested in developing new tetraploid varieties by crossing triploid varieties and wild or improved diploid clones with resistance to diseases. Some secondary triploids derived from crosses between these new tetraploid varieties and other diploid clones were also obtained. In the French West Indies, CIRAD has conceived another crossing strategy aimed at the development of triploid varieties directly from diploid plant material (Bakry et al. 2001).

Since the 1980s, apart from these conventional breeding approaches, other groups have focused on mutagenesis as at IAEA (Roux 2004) in Austria or on the selection of somaclonal variants as at TBRI (Hwang and Ko 1990) in Taiwan. These technologies appeared as a result of the development of in vitro culture techniques designed for rapid industrial multiplication of micro-propagated banana plants.

1.2 Botany and Origin

1.2.1 Morphological Description

Banana is a giant herb whose pseudostem, formed by interlocking leaf sheaths, reaches 1–8 m in height (Fig. 1.1). The leaves emerge from the apical meristem of the underground true stem, small in size, which is a corm or rhizome. The bud at

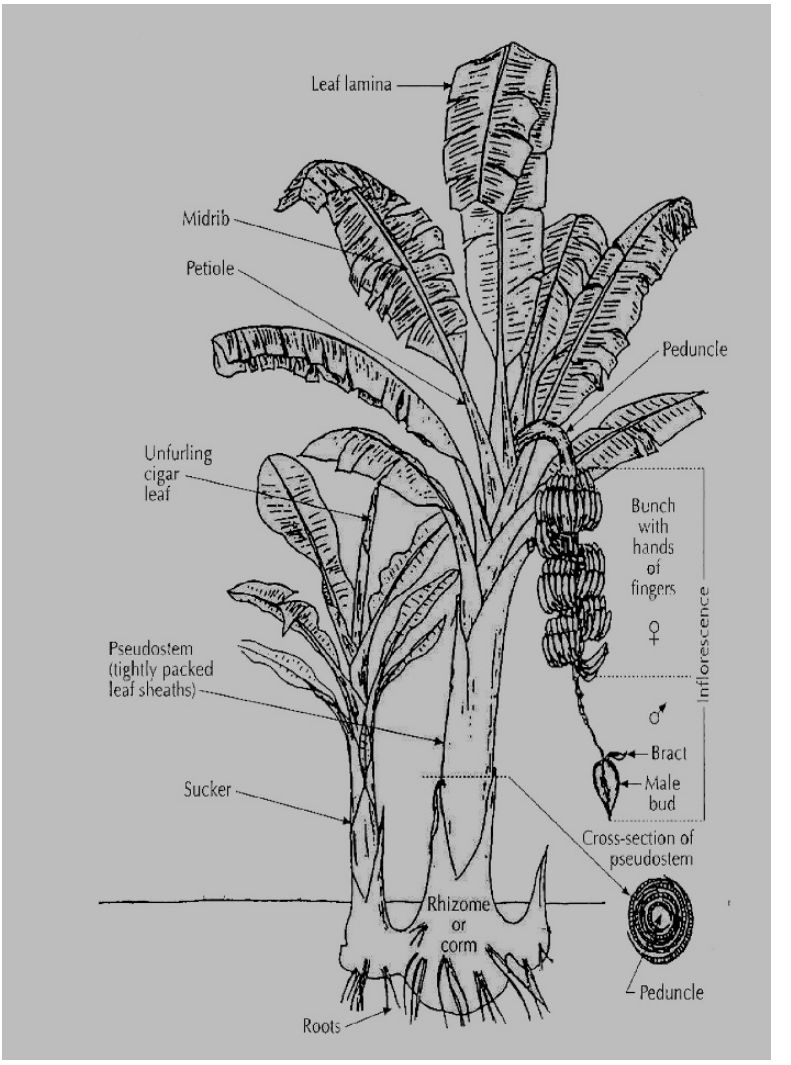

Fig. 1.1 Diagrammatic representation of a fruiting banana plant with suckers (from Champion 1963; Jones 1999)

the axil of each leaf eventually gives rise to a shoot. Shoot production is the natural reproductive mode for cultivated varieties. At the end of the vegetative phase, a quick change in the function of the central meristem induces the 'flower' primordia, followed by the growth and elongation of the true stem within the pseudostem and later by the emergence of the inflorescence.

The inflorescence, which can be vertical, pendant or sub-horizontal, is complex and made up of an ear of cymes. Cymes are inserted spirally on the floral stem and are composed of one spathe and single or double rows of flowers at its axils. These are the first ranks of flowers, usually called 'hands', from which the fruit bunches develop. The first hand contains flowers (termed female) with an ovary in the inferior position and non-functional stamens reduced to the state of staminodes. Sometimes the stamens develop, however, and these first flowers are hermaphrodite. Wild bananas have fruits filled with seeds but little pulp. In parthenocarpic banana plants usually called cultivars, the ovaries of female flowers are filled with pulp that forms the fruit without pollination or seed formation. As female fertility is quite low (and sometimes null), manual pollination is needed with cultivated varieties to get some seeds and, thus, progenies. After the female flowers, two or three hands of neutral flowers appear with undeveloped floral parts, followed by hands of male flowers (opposite the female flowers) with reduced undeveloped ovaries and well-developed stamens. In some cultivars, growth of the ear meristem is stopped very early (sometimes, immediately after *the* emergence of the first female flowers), but the inflorescence generally continues to grow indefinitely to form the so-called male bud. If it is not cut, this male bud will continue to grow until fruit maturity and stem withering. In addition to wild species, many cultivars have male flowers with some degree of pollen fertility.

1.2.2 Agromorphological Variations

Morphotaxonomy has made it possible to characterise different banana varieties and to establish the basis of the current botanical classification (Table 1.1; Simmonds and Shepherd 1955; Simmonds and Weatherup 1990). A set of 119 agro-morphotaxonomic descriptors has been defined as the norm for description of bananas (IPGRI-INIBAP and CIRAD 1996). These descriptors serve as a basis for a system of information exchange between collections, the MGIS (musa germplasm information system), run by INIBAP. Computerized tools have also been developed to identify the varieties on the basis of these descriptors (Perrier and Tezenas du Montcel 1990).

There is considerable variability regarding the aerial parts. Vegetative parts mainly vary with respect to pseudostem colour, the presence and colour of spots at the petiole base, the shape of the petiolar canal section and the plant height and growth habit. There may also be colour chimeras and variations due to dwarfism – obstruction or deformation of the inflorescences caused by highly compact interlocking of the leaf sheaths, stocky appearance of the leaves and shoot inhibition.

Table 1.1 Deduction of the genomic constitution of a variety based on its ploidy level and score (from Simmonds and Shepherd, 1955); 15 morphological characters were retained for their stability and capacity to discriminate among the different groups of cultivated bananas. Each character has been quantified on a scale of 1 to 5, in which 1 corresponds to a phenotypic expression of wild bananas of the species M. *acuminata*, called A, and 5 corresponds to that of wild bananas of the species M. *balbisiana*, called B. For each cultivar, the level of ploidy and the score obtained by the addition of notes for each of the 15 characters determine its genomic constitution and consequently its position in a given group

	Ploidy Level		
Theoretical score	2x	3x	4x
15	AA (16–23)	AAA (15–21)	AAAA (15–20)
30			AAAB (27–35)
35		AAB (26–46)	
45	AB (46–49)		AABB (45–48)
55			
60		ABB (59–63)	ABBB (63–67)
75	BB (69)		

The most important variations concern the inflorescences and consequently the fruit bunches. Differences between fruits are determined by their size, shape and colour along with the pulp colour. Clones of the plantain sub-group have a very firm cooking orange-yellow flesh, unlike other cooking bananas (sub-groups Laknao, Popoulou, Bluggoe and Monthan). Lujugira, the so-called East African highland bananas, are quite unique and, depending on the clone, used for cooking or brewing beer. Dessert bananas vary in taste and aroma: very sweet in some diploid Pisang Mas cultivars, sweet and acidulous in Silk Banana and bland in the universally appreciated export Cavendish bananas. Morphological variability in the male floral bud involves differences in the shape and colour of the bracts and male flowers.

Depending on the cultural conditions, the duration of the cycle is a varietal characteristic that is subject to wide variations. It ranges from nine to eighteen months, according to the variety, which is relatively critical in terms of the production potential of banana plantations.

1.2.3 Origin and Dissemination

Musa L. (Musaceae) is currently separated into five sections: Australimusa ($2n = 20$), Callimusa ($2n = 20$), Rhodochlamys ($2n = 22$), Eumusa ($2n = 22$) and Ingentimusa (unclassified species). Species usually classified among Callimusa and Rhodochlamys essentially contain plants of floral interest. Among the Australimusa, some accessions are cultivated for their fibre (abacá) and they belong mainly to *M. textilis*. Several other Australimusa accessions have edible fruit on erected bunches. Named Fe'i, they are only cultivated in the Pacific region. All the other *Musa* accessions with edible fruits are bananas.

As first suggested by Kurz (1865), Dodds (1943) and Cheesman (1947) show bananas related to Eumusa and originated mainly from two wild diploid species: *M. acuminata* (genome A) and *M. balbisiana* (genome B). Plants of these two species produce fruit filled with seeds. They reproduce both sexually and by vegetative means from shoots. In Southeast Asia, fruits of some wild accessions are consumed when immature before the seeds become hard and in particular varieties with soft seeded fruits. But real edible bananas are results of a combination of fruit parthenocarpy and sterility. As mentioned by Simmonds and Shepherd (1955), domestication is a succession of non-linear but interdependent stages: selection of parthenocarpic clones, selection for gametic sterility, selection of triploid plant and, finally, enhancement of the phenotypic diversity throughout vegetative propagation.

Parthenocarpy is usually considered as a pure *acuminata* character. It is described by Simmonds (1953) as polygenic. The domestication for starchy fruit was suggested to happen in the area from the Philippines, north of the Moluccas in Indonesia, to Papua New Guinea where some more starchy than usual seed-bearing wild *M. acuminata* subsp. *banksii* were observed by Simmonds (1962) and where wild accessions still have the cytoplasmic genome that is found in almost all cultivated bananas (Carreel et al. 2002). Whether parthenocarpic pure *balbisiana* exists is still being discussed (Valmayor et al. 1991; Jarret and Litz 1986).

Gamete sterility, supposedly with a genetic origin, has been described. Some are independent from parthenocarpy that yields variations in the morphology and physiology of the flowers (Dodds and Simmonds 1948; Dessauw 1988). Other factors have links with parthenocarpy and auxin metabolism that disturb the development of seeds in the fruits. Chromosomal factors also play a major role in banana fertility and, thus, in its evolution. Structural heterozygosity and triploidy are factors known to give meiotic errors that lead to lower fertility (Bakry et al. 1990). Dodds (1943), Dessauw (1988) and Shepherd (1999) showed that more than four chromosome rearrangements exist within the *M. acuminata* complex following the spatial and temporal isolation of the *acuminata* sub-species. Shepherd (1999) structured the species in six groups called 'translocation group' that differentiate by at least one rearrangement and he described the meiotic disturbance of the intergroup hybrids. He also showed that the sterility of inter-specific hybrids may have a genomic origin as the homology between the genome *acuminata* and *balbisiana* is partial.

Polyploidy in banana can be diploid, triploid or tetraploid. The 3*x* and 4*x* bananas are often more vigorous and give larger fruits than diploids. Most banana productions in the world rely on triploids while tetraploidy is considered as the maximum ploidy level giving usually viable plants with overall higher water content (personal observation), poor fruit post-harvest qualities and dropped leaves. Thus, triploidy is generally considered as the optimum ploidy level to have good agronomic behaviour as it guarantees the highest gamete sterility in production conditions. In natural conditions, triploid varieties have resulted from cross-pollinations between diploid clones producing 2*n* gametes and diploid clone producing *n* gamete (Fig. 1.2). The appearance of some tetraploids must have followed the same process ensuing the production of 2n gametes by triploid clones.

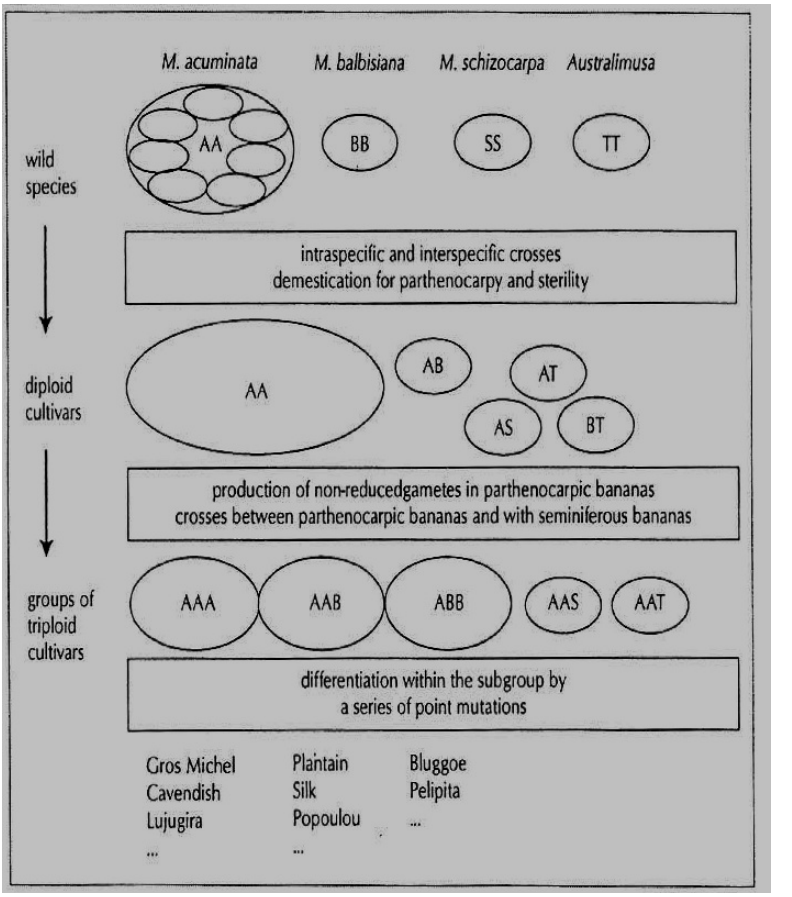

Fig. 1.2 Domestication of bananas

1.2.4 Enlargement of Phenotypic Diversity

The mode of reproduction of the triploid ancestral cultivars, characterised by a low degree of fertility, prompted natural somatic mutations, which contributed in the second phase, to the enlargement of phenotypic diversity. Thus, the global phenotypic diversity of the current triploid varieties resulted from two distinct phases: a first stage of fixation by the sexuality of ancestral triploid plants followed by a second stage of diversification due to the vegetative propagation of these proto-varieties by humans. In the *Musa* complex, varieties derived from each other by vegetative propagation are related to the same sub-groups.

Musa structuration, origin and migration of cultivars have been drawn by Champion (1967) and De Langhe (1995). These data are being clarified by linguistic (Rossel 1999) and phytoliths approach (Lentfer and Boyd 2004; Lejju et al. 2006; Ball et al. 2006). In the previous era, bananas were cultivated from India to the

Pacific region, from north of Australia to Taiwan and even in southern Japan. They were introduced at various times in Africa. More than 3000 years ago, plantains and probably a few diploids (still found in Comoro Islands today – personal data) were the first to reach East Africa through Pemba and Zanzibar, from Southeast Asia (De Langhe 1995). Bantu-speaking peoples took them to West Africa. Today, plantains have almost disappeared from the east coast of Africa, but are found in all the humid zones of Central and West Africa. In the fifth century, there was a second wave of introductions of so-called East African highland bananas: Mutika-Lujugira which are beer and cooking bananas originally from Indonesia that probably arrived via Madagascar.

On the American continent, the appearance of dessert-type banana plants is linked with the discovery of the New World in the fifteenth century. However, some authors have hypothesised that cooking types – plantains and popoulous – may have arrived earlier from the Philippines on the west coast of South America, in Peru and Ecuador, about 200 years before the current era (Langdon 1993). This early colonisation could explain the spread of banana in the eastern Pacific region, but these hypotheses remain controversial.

The evolution involved species existing within a given biotope. It gave rise to monospecific *M. acuminata* cultivars or to inter-specific hybrids derived from crosses between *M. acuminata* and *M. balbisiana* and even between sections Eumusa and Australimusa (Jenny et al. 1999).

1.3 Structuration of Genetic Ressources

1.3.1 Diversity of Diploids

The diploid bananas, wild and cultivated, are presently much less widespread than the cultivated triploids. However, they are still found in the endemic state throughout Southeast Asia. The plants are generally weaker and have smaller yields than the triploids. The diploid clones are nevertheless indispensable for genetic improvement programmes, especially because of the low fertility of the triploids. Deforestation and loss of traditional gardens endanger these precious genetic resources.

1.3.2 Wild Bananas

The seminiferous wild bananas of the genus *Musa* are found in the humid but well-drained valleys and glades of forests in the tropical zone, in south and Southeast Asia and in the Pacific, from the Indian peninsula to the Samoan islands. More than 25 species have been described and included within the genus *Musa*. Only those that have contributed to the genome of parthenocarpic bananas are discussed here.

Species belonging to the section Australimusa (giving the T genome) and *M. schizocarpa* (S genome) of the section Eumusa are present east of the range

of *Musa*: in the eastern area of Indonesia, Papua New Guinea and the Pacific. The Australimusa are identified by their erect inflorescence. The various species of this section were described by Cheesman (1947) and Argent (1976) as related and morphologically very close. Molecular analyses show little variability and structuration compared to the Eumusa section. *M. schizocarpa* is characterised by water-green stem colour and a green colour of the bracts of the male bud. Up-to-date little variability has been found at the morphological as well as at the molecular level compared to *M. acuminata* (Argent 1976; Carreel et al. 1994).

M. balbisiana, of the section Eumusa, is found from India to the Philippines, Papua New Guinea and occasionally in the Indochina peninsula. Up to the 1990s, accessions available in ex situ collections were showing a low variability and a structuration in four main types (Horry 1989). During the previous decade, new interest for this species resulted in the identification of more polymorphism (Uma et al. 2005; Ge et al. 2005). Even so, more plant prospection and molecular characterisation are still needed.

The extension of *M. acuminata*, section Eumusa, covers most of the area of distribution of the genus *Musa*, from west to east, from Myanmar to Papua New Guinea. The topography of its area of origin has led to geographic and, thus, reproductive isolation, which is a source of differentiation. Chromosome rearrangements (inversions and translocations) observed by Shepherd (1999) between different accessions of *M. acuminata* can be associated with this process of reproductive isolation.

The high level of morphological variation associated to its geographic distribution led to the description of nine *acuminata* sub-species (Fig. 1.3). This

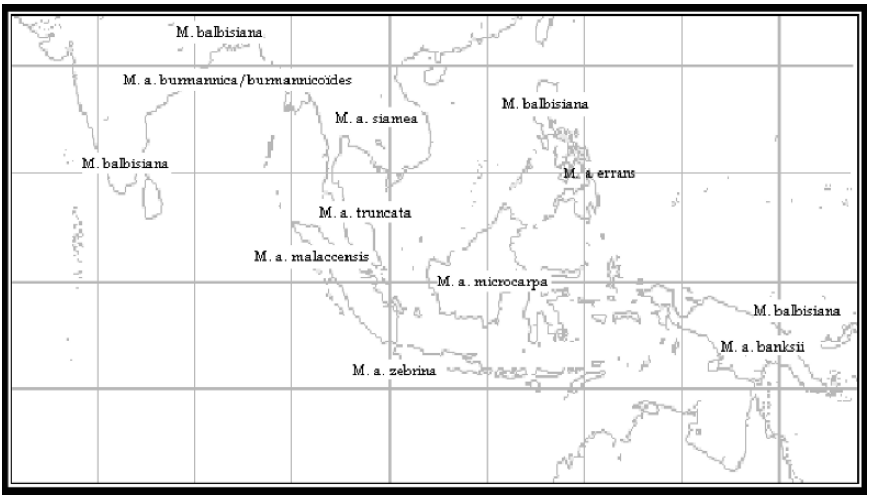

Fig. 1.3 Geographic distribution of Musa acuminata and Musa balbisiana in Asia

structuration has been described by cytogenetic, nuclear and cytoplasmic molecular analyses with the available accessions. It allowed to differentiate homozygote accessions from heterozygotes. Among the homozygotes, five pools can be defined. The first pool relates the *M. a. banksii* (Mueller) accessions from Papua New Guinea, the *M. a. errans* (Blanco) from Philippines and the *M. a. microcarpa* (Beccari) from Borneo area. It can be associated with the 'Standard structure' karyotype defined by Shepherd (1999) as what must have been the primordial chromosome structure. The second pool groups the *M. a. zebrina* (Van Houtte) from the western part of Indonesia and can be associated to the 'Javanese' karyotype of Shepherd. The third pool groups the *M. a. malaccensis* (Ridley) mainly from Malaysia and south of Thailand. The fourth pool relates to *M. a. burmannica* (Simmonds) and *M. a. burmannicoides* (De Langhe) from Bangladesh and eastern India to Myanmar, with *M. a. siamea* that originated in north Thailand and north Laos. This pool recognized by Shepherd as the Northern karyotype can be split in to two: Northern 1 and 2 that differ by only one translocation. Even if little prospected, the *M. a. truncata* is very distinct from all other sub-species (Carreel et al. 1994; Wong et al. 2001). This fifth pool must be associated to the 'Malayan Highland' karyotype of Shepherd though, unfortunately, no common accessions could be studied. From five pools to nine sub-species, it

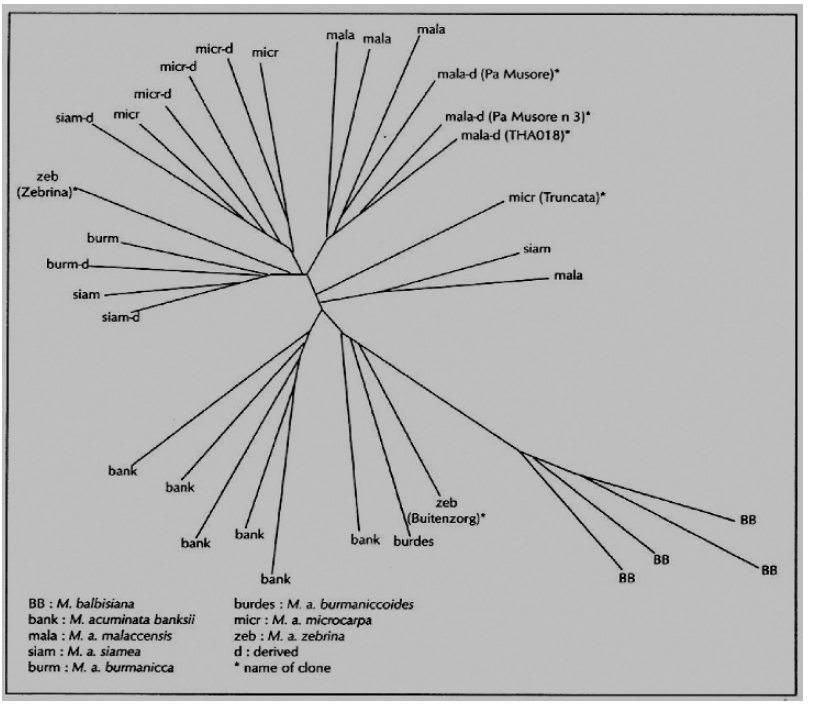

Fig. 1.4 Morphological diversity of seedy bananas (Guadeloupe collection): tree representation according to the NJ tree method, realized on the basis of dissimilarity between 32 accessions on the basis of 99 morphological descriptors (from Jenny et al. 1999)

shows how *M. acuminata* is variable and best-structured among the *Musa* species (Fig. 1.4).

1.3.3 Cultivated Diploids

Cultivated diploids are still mainly restricted to their area of origin in Southeast Asia. Nevertheless, some particular AA diploids have also been reported from the east side of Africa in Comoros (personal data) and Tanzania (De Langhe 2001). Only AA varieties of the Pisang mas (= sucrier) type, which have small, very sweet fruits, are cultivated on a large scale outside their zone of origin. Diploid edible bananas or cultivars are classified according to their genome in groups AA, AB, AS or AT, of which more than 90% are AA. For example, out of 135 diploid clones of the CIRAD collection in Guadeloupe (F.W.I.), only 10 accessions have been identified as inter-specific.

Among the AA, morphotaxonomic and molecular analyses revealed a nearly continuous cloud. Small sub-grouping only emerges for few accessions that are the most cultivated. Variability among those accessions belonging to the Pisang Mas type or Pisang Jari Buaya type is described as of somatic origin. The general cloud structuration can be explained, through the analysis of cytoplasmic and nuclear genomes, as originated from a gene flow between the *M. acuminata* sub-species. Carreel et al. (2002) showed that the AA cultivars are separated into four classes of chloroplastic profiles and five classes of mitochondrial profiles, each related to a specific sub-species. In banana, information on the chloroplast and mitochondrial genomes indicated a paternal heredity (Fauré et al. 1994) (Table 1.2). The AA cultivars can thus be divided into nine cytoplasmic types or cytotypes, but most of them correspond to three cytotypes. Each cytotype can be assigned either to distinct sub-species or to an inter-subspecific origin. That relatedness between cultivars and origin of the diploid cultivars has also been observed through the analysis of nuclear genome. From this classification, CIRAD has defined the base populations for its breeding programme (Bakry et al. 2001).

1.3.4 Triploid Bananas

The use of more complete morpho-descriptors or molecular markers and their analysis by multivariate statistical methods led to organisation in genomic groups: AAA, AAB and ABB with few AAT/ATT/AAS (Table 1.3). The RFLP molecular data do not indicate as clear a distinction between AAB and ABB as morphological markers. *M. balbisiana* is less polymorphic with the molecular markers and the two genomes B of ABB are rarely differentiated. Several AAB cultivars also have their two A genomes nearly identical. In these two cases, the AAB and ABB clones have molecular profiles of the AB type, and only a reading of the relative intensity of RFLP bands – which is possible only for some probe—enzymes combinations – allows us to clearly differentiate the AAB from the ABB (Carreel et al. 2002).

Table 1.2 Structuration of bananas according to their cytotype

Chloroplast profile	Mitochondrial profile								
	α	B	χ	δ	ε	φ	γ	η	τ
I									
II	*M. a. errans* AA cv (36)* AAA cv (Orotava, Red, Cavendish, Gros Michel) ABA cv (Mysore, Nadan, Pome)	AA cv (1)*	*M. a. burmannica* *M. a. burmannicoïdes* *M. a. siamea*	*M. a. malac-censis* AA cv (2)* AB cv ABA cv (Silk)	AA cv (1)*	AA cv (3)* AAA cv (3)**	*M. a. micro-carpa*		ABB cv (Saba 1, Bluggoe, Ney Mannan)
III	AA cv (4)* AAA cv (1)**				*M. a malac-censis*				
IV	AA cv (1)*		*M. a. siamea*						
V	AA cv (49)* AAA cv (4)** ABA cv (Laknao, Maia Maoli) ABB (1)*				AAA cv (Lujugira-Mutika)	*M. a. banksi* AA cv (18)* ABA cv (Plantain, Popoulou, Laknao)		AS w (2)*	ABB cv (Pelipita, Saba 2)
VI	AS cv (1)*					AS w (1)* AS cv (2)*		*M. schizo-carpa*	
VII									*M. balbisiana* Type 2
VIII	BAA cv (P. Rajah Bulu)				BAA cv (P. Kelat)				*M. balbisiana* Type 1, 3, 4 BAB cv (Peyan, Bendetta, P. Awack)
IX									BAB cv (P. Kepok)

* Number of clones having this cytotype.

** Number of clones of indeterminate sub-groups. In italics: seedy bananas. W: wild; cv: cultivated.

Table 1.3 Classification and geographic distribution of the principal banana cultivars

Sub-group	Cultivar	Type of fruit	Distribution
Group AA			
Sucrier	Pisang Mas, Frayssinette, Kirun	Dessert, Sweet	All continents
Pisang Lilin	–	Dessert	Indonesia, Malaysia
Samba	Samba, Chicame, Nzumoheli	Dessert, Acid	Comoros
Tjau Lagada	Tjau Lagada, IDN 110, Gu Nin Chiao, Sa	Dessert, Sweet	Indonesia
Sub-group AAA			
Cavendish	Lacatan, Poyo, Williams, Grande Naine, Dwarf Cavendish	Dessert	All continents (tropical and subtropical areas)
Gros Michel	Gros Michel, Highgate, Cocos	Dessert	All continents
Red	Red, Green Red, Pisang Glintong	Dessert	All continents
Lujugira-Mutika	Intuntu, Mujuba, Bwara, Nakitembe, Mukite	Beer and Cooking	East Africa (Uganda), Colombia
Ibota	Yangambi km5, Khom Bao, Pisang Saripipi, Lagun Vunalir	Dessert	Indonesia, Thailand, Africa
Group AB			
Ney Poovan	Ney Poovan, Safet Velchi, Lal Kelat	Dessert, Sweet Acid	India, Africa
Kunnan	Kunnan	Dessert, Sweet Acid	India
Group AAB			
Silk banana	Silk, Maçá, Malbhog, Supari	Dessert, Sweet Acid	All continents
Pome	Prata, Foconah, Dahomey, Pacovan, Pachanadan	Dessert, Sweet Acid	India, Malaysia, Australia, Brazil, West Africa
Mysore	Pisang Ceylan, Poovan, Zabi, Gorolo, Embul	Dessert, Sweet Acid	India, Sri Lanka, Malaysia, Comoros, West Indies, Zanzibar
Pisang Kelat	Pisang Kelat, Pisang Pulut	Dessert	India, Malaysia
Pisang Rajah	Pisang Rajah Bulu	Dessert, Cooking	Malaysia, Indonesia
Plantains	Dominico, Bobby Tannap, Batard, Orishele, Cuerno, Tanduk	Cooking	all continents
Popoulou/Maia Maoli	Iho U Maohi, Poingo, Popoulou, Maia Maoli	Cooking	Pacific (French Polynesia, Hawaii), Australia, Ecuador,
Laknau	Laknau, Adimoo, Bagatow, Mugus, Pisang Kastroli	Cooking	Philippines, Malaysia, Papua New Guinea
Pisang Nangka	Pisang Nangka	Cooking	Malaysia
Group ABB			
Pisang Awack	Fougamou, Bom, Pisang Kepok, Ducasse, Gia Hui, Muisa Tia	Dessert	Thailand, India, East Africa, Philippines

Table 1.3 (continued)

Sub-group	Cultivar	Type of fruit	Distribution
Bluggoe	Bluggoe, Matavia, Cacambou, Monthan, Barabay, Burro	Cooking	All continents (tropical and subtropical areas)
Pelipita	Pelipita	Cooking	Philippines, Latin America
Saba	Saba	Cooking	Philippines, Indonesia, Malaysia
Peyan	Peyan	Cooking	India
Group AAAA			
Champa Nasik	Champa Nasik	Dessert	- - -

The classification of triploid bananas is much easier to establish than that of the diploid clones because of the mode of evolution of the triploid clones. At this stage, there is almost no fertility and propagation is exclusively vegetative. By vegetative propagation, the clones are differentiated among each other only through small mutations that lead rapidly to the identification of true sub-groups. Thus, structuration of each group in sub-groups emerges from morphological analysis and the identification of morphotypes. This was confirmed by molecular markers: varieties belonging to the same sub-groups have identical or very similar nuclear and cytoplasmic profiles, these results expressing a very restricted genetic variability within each sub-group (Noyer et al. 2005). On the other hand, it is not true at the phenotypic level. The degree of variability within each sub-group is correlated to the intensity with which each type of clone was used and thus multiplied. The greatest phenotypic variability has been found in two sub-groups particularly exploited in Africa: plantains throughout the Central African zone and West Africa and Lujugira, also called the Highland East African Banana.

Molecular analysis also highlights few differences of classification usually due to homonymy. One of the most glaring examples is probably the Mnalouki cultivar, AAB of the Comoro Islands, the appearance of which causes it to be mistaken for a plantain of the French type: molecular markers and just the taste of the fruit, however, prove that it has nothing to do with the plantain. Differences between morphotaxonomy and molecular analysis also show that limits of sub-group are not always as clear as in the best known plantain or Cavendish sub-groups. D'Hont et al. (2000) checked with GISH the exact genome structure of some inter-specific cultivated clones. In most cases, the results were consistent with the chromosome constitution estimated by means of phenotypic descriptors (e.g. 11 A and 22 B for the ABB). It also shows exception may exist as for the clone 'Pelipita' that has 8 A and 25 B chromosomes instead of the predicted 11 A and 22 B.

Within each group, it is also possible to distinguish clones of the dessert type from clones of the cooking type within the AAB on a morphological basis, and within the three groups AAA, AAB and ABB by means of molecular markers (Fig. 1.5). Thus, among the AAB, dessert bananas of the sub-groups Silk, Mysore, Pome-Prata and Pisang Kelat are differentiated from the typical cooking bananas: Plantain, Popoulou, Maia Maoli and Laknao. It is to be noted that the clone Pisang

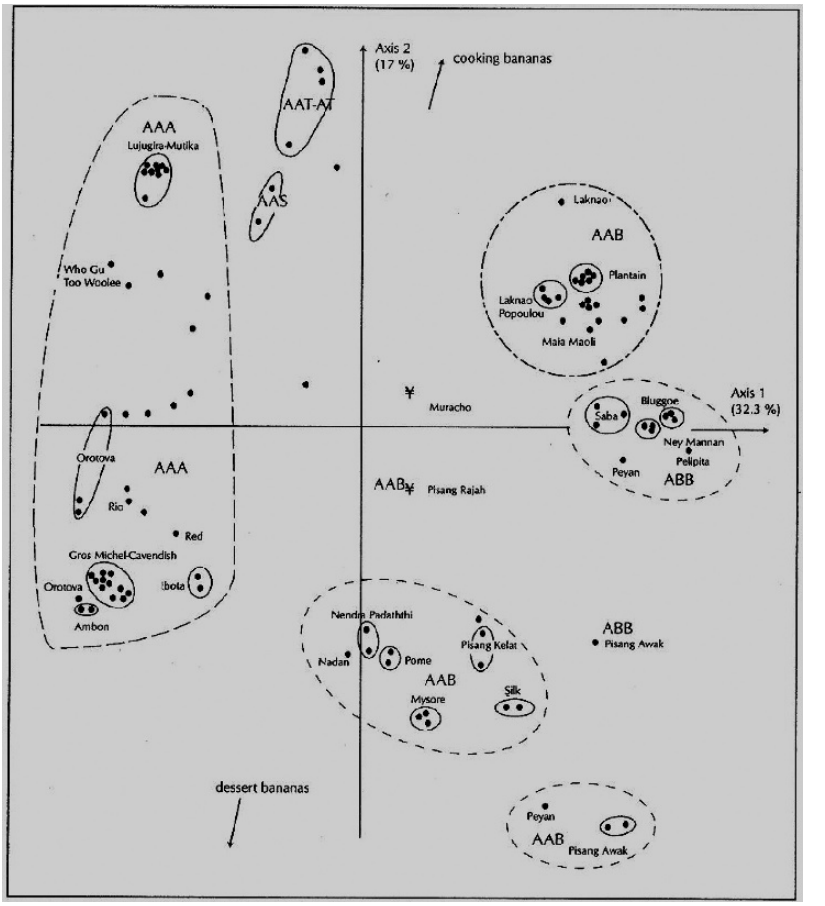

Fig. 1.5 Nuclear molecular diversity of triploid bananas according to their genomic group and sub-group. First plane of a factorial analysis on a Jaccard dissimilarity between 109 cultivars on the basis of 267 variables (from Jenny et al. 1999)

Raja Bulu of the sub-group Pisang Rajah has a profile intermediate between the dessert and cooking types This dessert/cooking classification can be ascribed to the genome A of each of these sub-groups.

1.3.5 Relationships Between Diploid and Triploid Varieties

Several morphological resemblances are known between diploid and triploid clones. Bunches of several AA cultivars – ‘Pongani’ and ‘Kekiau’, for example – that come from collections in Papua New Guinea are similar to those varieties of the plantain sub-group. The taste and type of consumption of other AA cultivars, such as ‘IDN110’, relate them to triploids of the Silk sub-group. These relationships have

Table 1.4 Identification of the putative diploid parents of cultivars from Cavendish and Gros Michel sub-groups

Attributes	Cultivars	Origin	Ploidy	Total number of bands	Bands in common with targeted triploid	Bands of targeted triploid not in 2*n* gamete	Pole 1 specific bands[a]	Pole 2 specific bands[a]	Pole 3 specific bands[a]
Targeted triploid	Gros Michel		3x	73	73	13	3	2	1
sub-group	Akondro Mainty	Madagascar	2x	60	60	–	3	1	0
Putative	Samba	Comoros	2x	60	60	–	3	1	0
unreduced	Chicame	Comoros	2x	60	60	–	3	1	0
gamete donor (2n gamete)									
Normal haploid	Sa	Thailand	2x	59	49	13	2	1	1
gamete donor	Khaï Naï On	Thailand	2x	59	50	13	2	1	1
(best matching	Fako Fako	Papuasy	2x	55	49	11	1	2	1
candidates)	Hom	Thailand	2x	52	39	11	0	0	5
Targeted triploid	Cavendish		3x	72	72	12	3	3	1
sub-group	Akondro Mainty	Madagascar	2x	60	60	–	3	1	0
Putative	Samba	Comoros	2x	60	60	–	3	1	0
unreduced	Chicame	Comoros	2x	60	60	–	3	1	0
gamete donor (2n gamete)									
Normal haploid	Sa	Thailand	2x	59	45	10	2	1	1
gamete donor	Khaï Naï On	Thailand	2x	59	46	10	2	1	1
(best matching	Pisang Rojo Uter	Indonesia	2x	54	48	9	1	3	1
candidates)	Pisang Bangkahulu	Indonesia	2x	59	45	9	1	2	0

[a] *M. acuminata nuclear RFLP genetic pole 1 assembles M. a. banksii and M. errans sub-species, pole 2 M. a. zebrina and M. a. microcarpa, pole 3 M. a. burmannica, M. a. burmannicoïdes and M. a. siamea, pole 4 M. a. malaccensis (Carreel 1994; Carreel et al. 1994). Specific bands are from one pole of wild diploid Musa acuminata germplasm that are not present in any of the other three poles.*

been confirmed by molecular analysis of cytoplasmic and nuclear genomes, and other relationships have been brought to the fore.

The emergence of triploids in *Musa* may be explained by the hybridisation between cultivars producing non-reduced gametes (Simmonds and Shepherd 1955) and diploids producing normal haploid gametes. For example, to trace the diploid ancestors of Cavendish and Gros Michel sub-groups, the nuclear RFLP patterns of 178 diploid clones representing the worldwide variability of the species were compared with that of the triploid varieties. This analysis led to the identification of mainly three AA clones (namely 'Akondro Mainty', 'Chicame' and 'Samba') as the common putative diploid ancestor of Cavendish and Gros Michel varieties that contributed to triploid formation through the production of 2*n* gametes. A sub-group of two Thai AA clones ('Sa' and 'Khai Nai On' suspected to be mutants of each other) was also identified as putative donor ancestor of the haploid gamete that may have brought the complementary alleles (Table 1.4; Raboin et al. 2005). This method can be applied to any mono- or inter-specific triploid clones.

Over 300 accessions, wild and cultivated clones, diploid or triploid, were studied with the same markers for its cytoplasmic and nuclear genome (Carreel et al. 1994, 2002). As for the Gros Michel and Cavendish sub-groups, it revealed relatedness between diploid and triploid accessions and highlighted putative ancestors of triploids. It allowed breeders to target the diploid progenitors that are more closely related to the triploids, which needs improvement.

1.4 Genetic Resources Utilization

For banana cultivation, a distinction can be made between production for export and for domestic markets, which are very important in areas such as India, Brazil and Africa where a subsistence food-crop system prevails.

Cultivation of bananas for export has passed through several stages in the last 100 years (Maillard 1986). It really began at the end of the last century, from 1870 in Jamaica where Baker organised the first exports of Gros Michel bananas to the North American markets and, in 1880, from Costa Rica where Keith set up a similar commodity chain. Two years later, Fyffe began to supply the English market with another variety, 'Dwarf Cavendish' from the Cavendish sub-group, which had flourished in the Canary Islands since the beginning of the fifteenth century. Fruits were first refrigerated for transport in 1903.

Techniques for commercial cultivation and large-scale exports were developed during this early period. Gros Michel, which is characterised by the natural robustness of its fruit, permitted shipment of entire packed bunches. Because of its tall height, only low-density plantations were possible – 800 plants per h – and treatment of plants against leaf diseases (carried out by spraying underneath the foliage) was difficult. This variety was perfectly amenable to the low-intensity agricultural practices of the time and areas cropped with banana continued to increase in Central America. Concomitantly, productivity decreased progressively in plantations due to plant wilt as the variety proved susceptible to a soil fungus, *Fusarium oxysporum*

f. sp. *cubense*, which blocks the conducting vessels of the stock and leaf sheaths. Identified in tropical America (Costa Rica and Panama) since 1890 (Stover 1962), this disease, called Panama disease, prompted growers to search for new territories as no effective antifungal treatment was known.

The quest for new territories ended in the 1960s due to a reconversion launched with varieties of the Cavendish sub-group that were resistant to Panama disease. Begun in 1945, this reconversion could only be achieved by reorienting cultural practices in favour of high intensification and changing the conditions of transport of fragile fruits. This led to a revision of North American market standards, which perforce had to accept a smaller banana. Intensive cropping systems had been adopted earlier by producers in the Canary Islands, French West Indies and Africa, which have opted for Cavendish bananas since the 1930s. At present, all bananas grown for export belong to the sub-group Cavendish in which the cultivars differ from each other only by mutations. Six main clones, distinguishable by their size and a few associated characteristics, have been cultivated for some years: 'Lacatan', 'Valéry', 'Poyo', 'Williams', 'Grande Naine' and 'Dwarf Cavendish'. Several new clones selected by industrial in vitro laboratories now complete this list. In poor crop management situations, producers tend to select large-sized varieties (over 4 m), which are less susceptible to leaf cluster blockage associated with water or nutrient stress. However, in well-managed agronomic conditions, preference is given to more productive varieties of intermediate size, such as 'Grande Naine' and 'Williams'. Although distinct from an agronomic point of view, all Cavendish varieties are difficult to differentiate by refined molecular biology methods. Production of dessert bananas for export (14 m tons per year in 2006) relies on a very narrow genetic basis and is therefore at the mercy of any new pathogen. In fact, this has already happened with the appearance of race 4 of *Fusarium oxysporum* f. sp. *cubense* on Cavendish bananas in subtropical production zones (i.e. South Africa, the Canary Islands, Australia and Taiwan).

More diversified production for local markets is estimated to be 87 m tons per year in 2006. The Cavendish bananas alone represent 43% of this domestic production, the other sweet and sweet acid varieties totalizing nearly 12%. Among cooking bananas, plantains represent 18% of the world production whereas other cooking varieties and bananas of mixed use totalize 26% (Lescot 2006). Many banana cultivars, genetically very different, are cultivated in Southeast Asia and the Pacific region. Diversity within the same area – field, plot, farm – reduces with remoteness from the centre of origin of the species. Dessert banana production and consumption in Brazil, one of the three largest producing countries in the world, together with India and Uganda, are mainly based on varieties belonging to the Cavendish, Pome and Silk sub-groups. In West and Central Africa, production is based on cooking bananas of the plantain sub-group, including more than 100 cultivars. Beer and cooking bananas, different from plantains, are chiefly grown in East Africa. Europeans and North Americans almost solely consume Cavendish bananas. Contrarily, Asians relish all types of bananas: 'Pisang Mas' (the small 'Figue Sucrée'), Pisang Awak and Lakatan, among the better known, but also Pisang Tandok of the plantain sub-group, Gros Michel and Silk bananas.

1.5 Current Status of Breeding Efforts

1.5.1 Breeding Objectives

Since the 1920s, priority in all genetic improvement programmes has been given to the development of varieties resistant to diseases and pests. The secondary objectives are more specifically linked with socio-economic aspects of production and natural susceptibilities of varieties. For export bananas, studies have been oriented towards early flowering, highly productive varieties, with perfectly cylindrical fruit bunches and uniform-sized fruit as this facilitates their packaging and marketing. For domestic markets, varieties need to have high tolerance to biotic and abiotic stresses. They must have strong root systems to enable good soil anchorage and effective uptake of water and mineral resources. Finally, they should be able to withstand primitive transportation and preservation conditions. Preference should be given to small-sized varieties that are less sensitive to gusts of wind that can break the pseudostems and even uproot the plants (Table 1.5).

Concomitantly, recent advances in molecular biology and in tissue culture have opened new avenues (tissue culture techniques including protoplast somatic hybridisation and genetic modified organism) to meet breeding objectives (Hwang and Ko 1990; Remy et al. 1998; Haïcour et al. 2004; Smith et al. 2006). Introduction of exogenous genes in current varieties will probably enable breeders to obtain varieties resistant to viruses in the near future. In another sphere, after obtaining successful results in tomato, research is now underway on developing banana varieties with slower fruit ripening in order to facilitate marketing. Finally, some research groups envisaged the utilization of genetically transformed bananas for vaccinating humans in the developing countries (Sala et al. 2003).

1.5.2 Breeding Programmes

Several important research centres are interested in the selection of the banana tree.

FHIA, Honduras: The overall objective of the programme is to develop hybrids of bananas and plantains that are resistant to the main diseases and insects of economical importance. The resulting hybrids are also screened to determine the best ones that have the ability to prosper under adverse growth conditions. The ultimate goal is to reduce the dependency of these agricultural crops on pesticides and chemicals and to contribute to the sustainable development of these crops with regard to both production and productivity. FHIA has already made available internationally the following tetraploid hybrids: FHIA-01, FHIA-02, FHIA-03 and FHIA-21. These hybrids are now positively contributing towards food production in various countries throughout the world. FHIA-01 and FHIA-02 are being introduced into export markets of organic products. The main characteristic of these hybrids is their resistance to various diseases that have recently devastated plantations throughout the world including BLSD, *Fusarium* wilt and Bacterial Wilt everywhere.

Table 1.5 Breeding objectives for bananas

Sub-group	Regions of production	Cropping strategy	Breeding objectives
Cavendish (AAA) Dessert type	Latin America, Caribbean, Philippines, India, West Africa, Mediterranean countries	Export banana intensive system, Local markets	Resistance to diseases (Black Leaf Streak, Sigatoka, *Fusarium* wilt race 4), nematodes, weevils. Slow fruit ripening. Tolerance to drought and cold temperature.
Silk and Pome (ABA) Dessert type	Brazil, India, Australia, Southeast Asia	Local and regional markets, Food crop system Extensive system, Intensification in progress	Resistance to diseases (Black Leaf Streak, Sigatoka, *Fusarium* wilt), nematodes, weevils. Fruit quality (fragility). Adaptation to cold temperature.
Bananas of East Africa (AAA) Dessert and beer types	East Africa	Local market, Food crop system	Resistance to diseases (Black Leaf Streak, Sigatoka, *Fusarium* wilt), nematodes, weevils.
Plantains (ABA) Cooking type	West Africa, India, Latin America	Local and regional markets, Food crop system, Intensification in progress	Resistance to diseases (Black Leaf Streak), nematodes, weevils. Productivity. Sucker production.
Popoulou/Maia Maoli (ABA) Cooking type	Pacific	Local market, Food crop system	Resistance to diseases (Black Leaf Streak, *Fusarium* wilt).
Saba, Bluggoe (ABB) Cooking type	Southeast Asia, All marginal zones, Latin America, Carribean	Local market, Food crop system, Processing industry	Resistance to *Fusarium* wilt, Moko disease and nematodes.

EMBRAPA-CNPMF, Brazil: One of the greatest problems in banana cultivation in Brazil is the lack of productive commercial varieties, with adequate plant height and resistance to the main pests and diseases. In addition to the diseases that already occur in Brazil, such as *Fusarium* wilt, Yellow Sigatoka, Bacterial wilt, nematodes and insects, it is mainly the rhyzome weevil borer that damages the crop. BLSD, recently introduced, can cause even more damage to the Brazilian banana cultivation. Its objective is to develop varieties resistant to insects and nematodes, reduce plant height and cycle and increase yield. The banana genetic breeding programme conducted in Brazil is based mainly on the production of AAAB tetraploids by crossing improved diploids (AA) with Prata (Pome) and Maçã (Silk) triploid bananas (AAB). The main obtentions of EMBRAPA are three new sweet varieties: Pacovan Ken – AAAB, Preciosa – AAAB and Tropical – AAAB.

IITA, Nigeria, Cameroon and Uganda: The diversity and sustained production of the banana crop in East and Southern Africa is threatened by pest and disease problems. Banana weevil and a complex of parasitic nematodes are the major pests, while fungal leaf spots, *Fusarium* wilt and viruses are the most important diseases. Shortening fallow periods and a general decline in soil fertility as a consequence of increasing population pressure on available land resources further compound these production constraints. The banana improvement programme pursues a holistic approach, combining disease-resistant cultivars and integrated pest management (IPM); including clean propagules and biological control and better crop husbandry techniques for stable and sustainable production. The initial steps for genetic improvement of plantains or bananas traditionally involved crossing the $3x$ accessions to be improved with a $2x$ accession that is disease-resistant to produce $4x$ hybrids. The $4x$ hybrids are both female and male fertile that often reduces fruit quality due to the presence of seeds in the pulp, which is overcome by crossing the $4x$ selections with $2x$ accessions to produce secondary $3x$ hybrids. Recurrent diploid breeding to make $2x$ strains with better resistance or other desirable traits completes this genetic improvement process. IITA released primary starchy tetraploids (BITA 03 – AAAB; PITA 14 – AAAB) and secondary starchy triploids (PITA 16- AAB; PITA 21 – AAB; PITA 23 – AAB). BITA 03 is also used for production of wine and PITA 16 for the production of beer in East Africa.

CARBAP, Cameroon: In West Africa, banana and plantain production is severely threatened by several pests and diseases. Furthermore, wind damage severely affects yield. Based on the large number of accessions collected in the primary and secondary zones of banana diversity, the initial breeding approach was focused on triploid × diploid crosses ($3x/2x$) aimed at producing disease-resistant tetraploid hybrids from partially fertile AAB cooking varieties. Moreover, this $3x/2x$ approach has led to the creation of diploid hybrids that are used to develop elite diploids, among other breeding strategies. The main varieties released by CARBAP today are natural cooking varieties such as Pelipita (ABB) and cooking type hybrids such as CRBP39 (AAAB).

TNAU, NRCB and BRS, India: India is endowed with rich varieties of bananas and plantains. The commercially important cultivars are 'Robusta' (Cavendish, AAA), 'Red banana' (AAA), 'Rasthali', also known as 'Silk' (AAB),'Poovan', also known as 'Mysore' (AAB), 'Virupakshi', also known as 'Lady finger' (Pome, AAB), 'Nendran', also known as 'French plantain' (AAB), 'Karpooravalli', also known as 'Pisang awak' (ABB), 'Monthan', also known as 'Bluggoe' (ABB) and 'Ney poovan' (AB). In India, banana production constraints are Sigatoka leaf spot diseases (SLSD), *Fusarium* wilt (race 1 and race 2), viral diseases (BBTV, BBMV, BSV and CMV), nematodes (*Pratylenchus coffeae, Radopholus similes* and *Meloidogyne incognit)* and weevil and borers (*Compolites sordidus* and *Odoiporus longicollis*). Improvement of bananas incorporates both conventional and new breeding techniques. Recurrent breeding was undertaken at the diploid level and many synthetic diploids with drought tolerance, resistant to BLSD and *Radopholus similis*, have been developed. Crosses between triploids and diploids have resulted in the development of tetraploids. Evaluation of these hybrids for yield, fruit quality

and resistance to nematodes, *Fusarium* wilt and leaf spot disease has led to the identification of potential hybrids. Two improved hybrids BRS-01 and BRS-02 have been developed at BRS, Kannara and evaluated at NRCB. The tetraploid BRS-01 Pome hybrid (Agniswar × Pisang Lilin) is highly *parthenocarpic* and sterile and exhibits immunity to *Mycosphaerella*, *M. eumusa* and *M. musicola* with an average bunch yield of 25 kg. The BRS-02 hybrid is another tetraploid (AAAB) from Vannan × Pisang Lilin of Mysore type exhibiting tolerance to *M. musicola* and *M. eumusae*. It has an average yield of 30–32 kg with 12 months of crop cycle. Both have been tested at the All India Co-ordinated Research Project (AICRP) on Tropical Fruits and released for commercial cultivation in the state of Kerala. In addition, in vitro mutation breeding by using gamma irradiation and chemical mutagens has resulted in the creation of superior mutants of 'Robusta' with good bunch traits. After these first results, the breeding activities have been reoriented towards the triploid strategy. In vitro polyploidization of Indian diploid clones has resulted in the synthesis of doubled-diploids that are currently evaluated for their breeding potential.

CIRAD, France: For the past few decades, the CIRAD in Guadeloupe strongly invested on the improvement of banana cultivation with emphasis on production, disease resistance and export trade. This strategy, proposed by CIRAD, is based on the synthesis of triploid hybrids coming from crosses between diploid and doubled-diploid ancestral varieties. This procedure is based on good knowledge of the evolution of the genus *Musa* leading to the present natural varieties. Some new synthetic AAB hybrids (IRFA909, IRFA910 and IRFA914) were released, but their diffusion had to be stopped because of the appearance of plants infected with a badnavirus. It was found that these infections resulted from the activation, following crosses, of BSV sequences incorporated in the genome of the *M. balbisiana* parent (see Section 1.5.3). Efforts are now concentrated on the creation of AAA hybrids using the species that does not contain activable integrated sequences. New triploid hybrids, resistant to the major diseases, have recently been obtained by crossing. Two new productive AAA sweet varieties (FLHORBAN 920; FLHORBAN 918) showing export potential and resistance to SD, BLSD, *Fusarium* wilt and nematodes have been released to banana growers in the French West Indies.

1.5.3 Constraints to Banana Breeding

Cultivated bananas are mostly triploid, with a low fertility and high heterozygosity. Progenies are usually of small size and composed of a mix plant of different ploidy level or aneuploids. Little knowledge on genetics and heredity is available.

The low fertility of cultivated bananas is a handicap for breeders. Triploid varieties may usually produce a few seeds when pollinated. In triploids, seed setting is overall higher in ABB in comparison than AAB. As reported by Simmonds (1987), in Malaysia, Pisang Awak (ABB) cultivated in the backyards of homes is well known as being frequently so full of seeds as to be almost inedible. In controlled conditions, seed set is lower and may vary from no seed in Cavendish to

a maximum of 400 seeds per bunch in some Popoulou AAB clones when pollinated with wild species (Bakry and Horry 1992a). In triploid varieties, the number of fruits per bunch varies from 100–250 and the number of ovules ranking from 300–600 per fruit. In the absence of gamete sterility, the seed potential could be theoretically estimated from 30,000 to 150,000 seeds per bunch. Consequently, it may be estimated that in triploid bananas, reproductive barriers induce a reduction of about 99.8–99.9% of female fertility. In diploids, the inter-specific AB clones are completely sterile probably (due to the partial homeology between the *acuminata* and *balbisiana* chromosomes) whereas AA varieties show a wide range of male and female fertility. Nevertheless, the overall fertility of AA varieties is higher than the triploid fertility. In wild clones, there are fewer reproductive barriers, the male flowers being plenty of viable pollen as the fruits, plenty of seeds. Quantities over 5,000 seeds per bunch are usual in wild *acuminata* as wild *balbisiana* accessions.

Beyond scarcity, seeds in cultivated clones are often abnormal with the absence of embryo or endosperm and sometimes both. For instance, absence of endosperm is very frequent in inter-specific *acuminata* × *balbisiana* crosses. Moreover, seeds containing no endosperm usually show irregularities compared to wild clones. So, the seeds coming from controlled pollinations germinate rarely (over 20%) under greenhouse conditions. This explains why the in vitro embryo rescue is systematically used in banana breeding, which can increase germination rate often up to 85% (Bakry and Horry 1992a).

These difficulties to breed cultivated bananas did not favour progress in genetic studies. Abnormalities in the meiosis are very frequent in diploid and triploid clones showing chromosomes pairing in multivalent in place of regular bivalents (Shepherd 1999). At the diploid level, structural heterozygosity that is present in most diploid varieties brings aneuploid gametes with 12–16 chromosomes instead of 11 (personal observation). These irregularities lead to the formation of gamete distributions with strong shifts for recombination and in relation to the random distribution of alleles (Vilarinhos 2004; Vilarinhos et al. 2004). At triploid level, occurrence of aneuploid gametes and formation of 2*n* to 4*n* gametes are also frequently reported. All these abnormalities complicate the understanding of genetic segregation in the progenies. So far, no genetic studies have been really developed on bananas. For the moment, the principles of banana improvement still rely on crosses between two parents bringing complementary features followed by a phase of phenotypic selection before eventual new crosses. The breeding strategies in bananas are not yet based on the recombination of genes but more on the association (or reassociation) of outstanding phenotypes.

More recent constraints in banana breeding are related to the occurrence of banana streak disease (BSD) in progenies caused by several strains of BSV (a plant pararetrovirus, genus *Badnavirus* from the Caulimoviridae family). Many inter-specific *acuminata/balbisiana* hybrids derived from parents free of BSV have been found infected with one or several BSV strains that may lead to complete death. This infection is thought to arise from viral integrated sequences (EPRV, endogenous pararetrovirus) in the nuclear genome of *M. balbisiana* (B genome) (Harper et al. 1999). Some of the plants are immediately infected by virus after crosses (Lheureux

et al. 2003). Other hybrids showed a later expression of the disease under stress conditions (e.g. in vitro propagation [Dallot et al. 2001] or unfavourable conditions such as cold temperature). The mechanism by which the EPRV-BSV is activated in inter-specific hybrids is under investigation.

Some investigators decided not to use donors of B genome as they were shown to increase the probability of triggering the activation of EPRV-BSV. It is unfortunate because *M. balbisiana* confers rusticity, hardiness, good ratooning and ability to produce strong root system in hybrids. Till now, no *M. balbisiana* accession free of EPRV-BSV has been found in the most important ex situ *Musa* collections around the world. Therefore, there is a huge necessity to prospect new endemic *M. balbisiana* accessions in the centre of origin of the species (Uma et al. 2005) and to determine their status regarding BSV integrated sequences. At the same time, it is necessary to initiate a breeding programme to free the B genome from EPRV-BSV susceptibility.

1.6 Breeding Strategies and Methodologies

In spite of inherent gametophytic sterility, notable progress has been made over the last 20 years. Two radically different strategies have been adopted to develop tetraploid and triploid varieties. The first rely on the synthesis of primary tetraploid hybrids, eventually followed by a second phase of secondary triploid synthesis. The second strategy relies on the synthesis of triploid hybrids directly from diploid germplasm.

1.6.1 The 3x/2x Strategy: Development of Primary Tetraploids and Secondary Triploids

From the beginning, breeders tried to exploit triploid varieties showing residual fertility when pollinated with a diploid clone. In spite of their high degree of sterility, some triploid varieties produce few seeds. Among them, some arise from a few unreduced triploid eggs cells ($n = 3x = 33$) that could be fertilised with normal haploid pollen to give tetraploid embryos (Fig. 1.6; Bakry and Horry 1992a). The value of this phenomenon is that genes from the mother plant do not segregate and, therefore, the maternal characteristics are retained that made the fruit marketable. The breeders conceived the idea of adding one set of chromosomes from a diploid carrying heritable resistance to disease to the three sets from the mother (Fig. 1.7; Menendez and Shepherd 1975).

The occurrence of phenotypic segregation in the tetraploid progenies from plantains (Ortiz and Vuylsteke 1998) or from Popoulous (Bakry and Horry 1992a) is important regarding the mode of production of $2n$ eggs (Table 1.6; Sanford 1983; Veilleux 1985). The fact that heptaploid hybrids in progenies can be obtained from some triploid varieties (Prata-AAB and Poingo-AAB) clearly suggests the formation of $4n$ eggs in *Musa* arising from a first-division restitution (FDR)

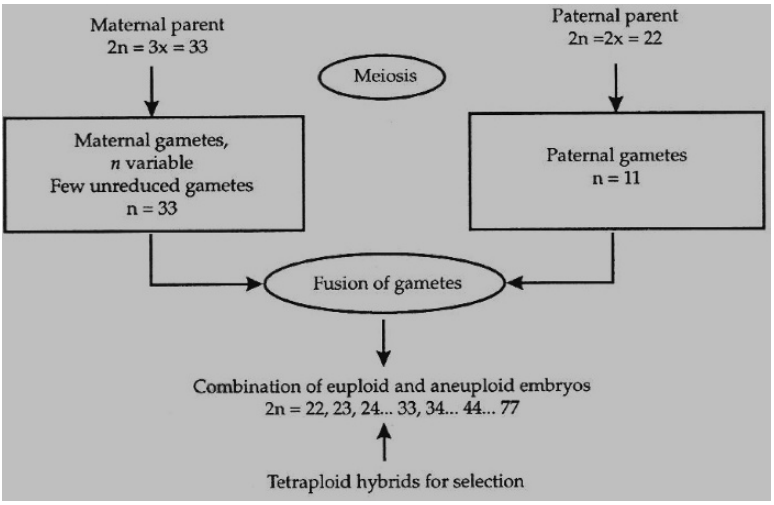

Fig. 1.6 Development of tetraploid varieties by restitution of maternal gametes in triploid varieties

and from a second-division restitution (SDR) of the mother parent. Moreover, as reported by Dodds and Simmonds (1946), apospory may occur in *Musa*. In practice, one can observe that tetraploid hybrids are often very close to the female parent, suggesting a transfer of the maternal heterosis via FDR to progenies. At the opposite, some tetraploid hybrids clearly show a dramatic decrease of vigor in comparison to the triploid mother plant (personal observation) indicating SDR derived 2*n* gametes.

This strategy of breeding was developed back in 1922 to confer *Fusarium* wilt resistance to cv. Gros Michel and taken up again with other varieties of dessert and cooking bananas to confer resistance to the BLSD and nematodes. The dessert tetraploid hybrids from FHIA arose from the pollination of dwarf mutants of 'Gros Michel' and 'Prata' with improved diploids resistant to BLSD and SD and to the nematode *Radopholus similis*. As for the cooking bananas, CRBP-39 from CARBAP, FHIA-21 (plantain hybrids) and BITA 3 from IITA (a cooking banana hybrid) arose from crosses between a plantain variety and a diploid clone resistant to BLSD.

It should, however, be noted that nearly all the AAAB hybrids resulting from crosses between an AAB and an AA can show symptoms of Banana Streak disease caused by the activation of viral sequences integrated in the B genome of these cultivars. Furthermore, the low fertility of the rare fertile cultivars means that only small populations can be generated and a considerable number of crosses have to be made to have a chance of generating useful results. Another problem is the high water content in tetraploid fruit, which ripen and soften rapidly. Moreover, tetraploid hybrids – AAAB as well as AAAA and AABB – are often much more fertile than the triploid mother plant. Consequently, they are liable to produce fruits containing some seeds if they are pollinated, which obviously reduces their quality. Removing the flowers avoids unwanted pollination. One must also notice that the $3x/2x$

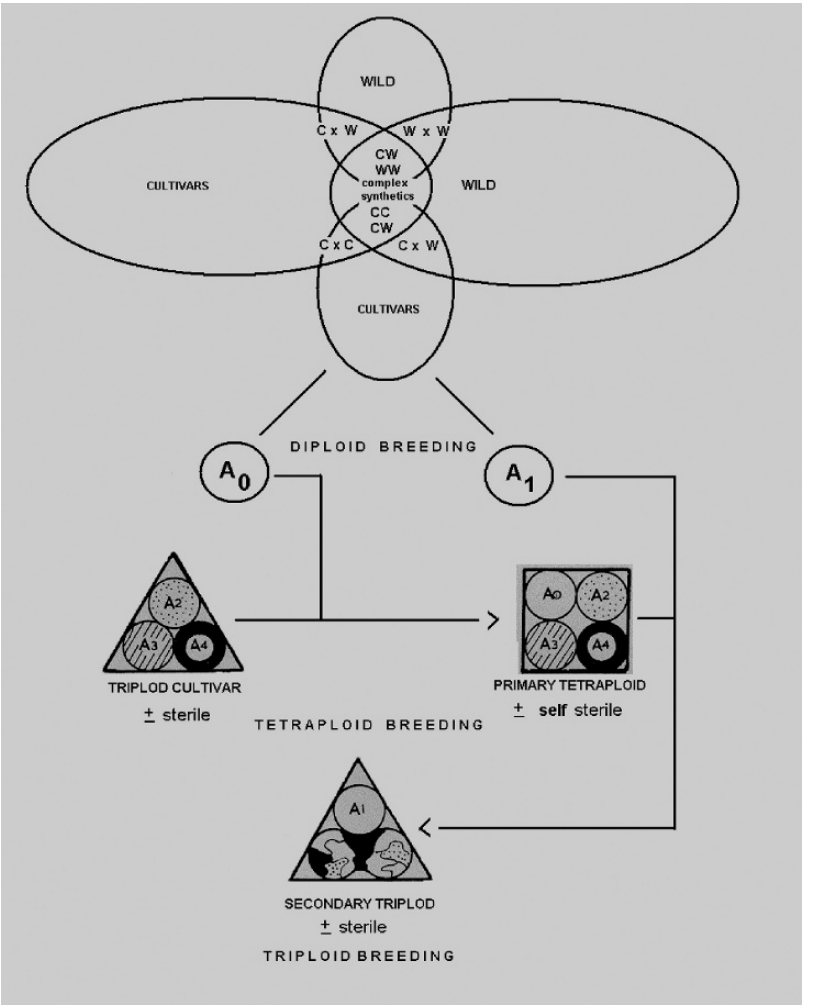

Fig. 1.7 Diagram of banana breeding in the 3x/2x strategy based on M. acuminata (useful derivatives only shown). A0 to A4 = individuals sets M. acuminata genes (from Menendez and Shepherd 1975)

strategy also enables numerous types of AA diploid hybrids to be created. More than 50% of the seedlings generated by certain plantains are AA diploids.

1.6.2 Improvement of the Diploid Male Parents

The choice of the diploid parent is obviously crucial. Since the FDR triploid eggs of the maternal parent are rather homogenous, all breeding efforts should be based on the improvement of the diploid male parent.

Table 1.6 Percentage of parental heterozygosity transmitted through gametes arising from different mechanisms. The beginning material in each case is assumed to be diploid (from Sanford 1983)

Origin of gamete	Parental heterozygosity (%)
Apospory:	100
Asynapsis plus first division restitution:	100
First division restitution:	75–83[a]
Somatic doubling followed by normal meiosis:	66.6
Second division restitution:	33–50[a]
Post-meiotic doubling:	0
Normal reductional meiosis:	0

[a] Depending on chromosome morphology and crossing-over patterns.

The great genetic diversity of the diploids collected in Southeast Asia and India has led to the selection of wild, semi-*parthenocarpic* and *parthenocarpic* diploids such as 'Calcutta 4' (*M.a.* ssp. *burmanicoïdes*). But, wild seedy diploids, amongst which resistance to *Fusarium* wilt and SLSD occur, have very few of the outward appearances of commercial bananas. Their fingers are short, seedy and their bunches are rarely pendulous. *Parthenocarpic* edible diploids showing often a slight male fertility have a better shape and larger fruits, but all are not resistant to diseases. Consequently, intensive breeding at the diploid level has been developed these favorable agronomic features in a single genotype. Notable among these is M53 (resistant to SLSD and to *Fusarium* wilt), bred in the 1950s by the breeding programme of the former Jamaican Banana Board. Elite diploids have also been selected by FHIA (Rowe and Rosales 1993), some of which have multiple resistance (to SLSDs, nematodes and *Fusarium* wilt) and markedly longer fruit than the small fruit of wild diploids. Moreover, numerous mono-specific AA and inter-specific AB diploid hybrids have been created by the NRCB and the TNAU in India.

More recent is the use of fertile diploid AA hybrids (resulting from $3x/2x$ crosses) as parents or as starting material for developing elite diploids. In particular, these are plantain hybrids resistant to SLSDs and with a fruit quality similar to that of plantains. This approach is used especially by CARBAP and IITA to develop diploids specific to certain sub-groups such as plantains and the East African highland bananas.

1.6.3 Synthesis of Secondary Triploids from Tetraploid Hybrids

In view of the problems associated with the $3x/2x$ strategy and the fertility of the tetraploids thus obtained, the latter were soon regarded as intermediate products for the synthesis of secondary triploids when crossed with diploids. This material has been exploited on a large scale using fertile tetraploids and diploid hybrid parents (F_1 hybrids) capable of generating larger populations of F_2 hybrids that are in fact the grandchildren of popular triploid cultivars. However, one should not lose sight

of the fact that the genetic gain obtained by nuclear restitution of the preceding stage (i.e. the transmission of heterosis from the triploid variety to the tetraploid hybrid through FRD) will be lost by the redistribution and the recombination of the alleles at each locus that will occur during meiosis of the tetraploid hybrid (Fig. 1.7).

The synthesis of secondary triploids has been developed with some success in cooking bananas. Because of high homozygosity of their *acuminata* genome, the cooking bananas are likely to be low in genotypic variance and the inheritance of major agronomic traits is probably resultant of additive gene effects. Indeed, this strategy has allowed IITA, CARBAP and FHIA to obtain progenies of sufficient size to make some selections in secondary triploid hybrids. CARBAP used this approach to generate some dwarf-type triploid hybrids obtained from primary tetraploid hybrids that themselves arose from triploid cultivars of dwarf plantain. The main advantage of this two-stage strategy is to obtain, via primary tetraploid hybrids, several hundred second generation descendants from very sterile triploid cultivars.

1.6.4 Synthesis of Triploid Hybrids from Diploid Germplasm

CIRAD developed a new breeding strategy aimed at the creation of triploid hybrids directly from diploid germplasm. It is also noteworthy that all the natural varieties having a strong economic impact at the world level were triploid, triploidy giving in banana a selective advantage over the other ploidy levels. This breeding strategy is based on a search of specific combining ability between two diploids of which one is donor of diplo-gametes. As the production of 2*n* gametes is uncontrolled and fairly rare in diploid clones (Dodds 1943), the regular production of diplo-gametes has been achieved through the chromosome doubling of diploid varieties (Bakry and Horry 1994; Bakry et al. 2001).

By complementarities, this approach aims to associate the favourable traits brought by both parents and to maximize the heterozygosity in the triploid progenies (Table 1.6; Sanford 1983). Focus has been given on the identification of diploids that may be involved in the natural triploid varieties (Raboin et al. 2005). After a primary phase of agronomic evaluation and fertility testing, mono- and inter-specific diploids were selected and treated in vitro with colchicine for chromosome doubling to form auto- or allotetraploids (Fig. 1.8). Genotypes intended for colchicine treatment were selected according to the type of banana to be developed (cooking or dessert), their agronomic characteristics, their behaviour with respect to diseases and their paternal and/or maternal fertility. Improved diploids obtained by hybridisation and expressing an high resistance to various diseases are also included in this treatment. Over 25 AAAA autotetraploids and AABB allotetraploids have already been obtained (Bakry et al. 2007). All these plants have flourished in tropical conditions in Guadeloupe. Reciprocal crosses with diploid clones were soon undertaken to investigate the synthesis of mono- and inter-specific triploid hybrids.

The ability to set progeny is strongly linked to gamete fertility that is highly variable from clone to clone. The gamete fertility of the doubled AA diploids is

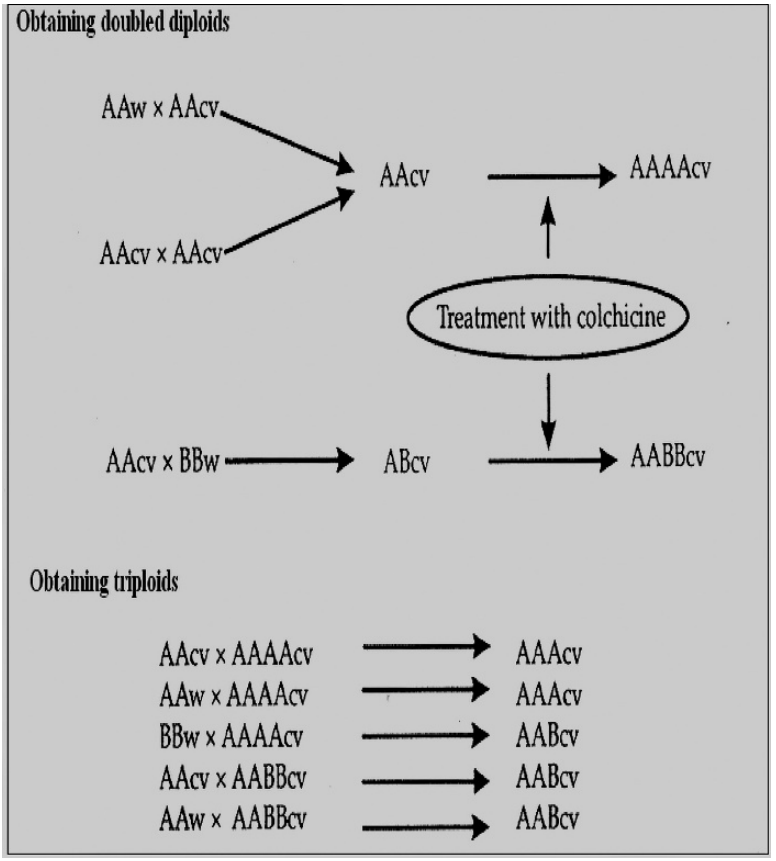

Fig. 1.8 Scheme for the synthesis of triploid varieties from diploid clones. w – wild; cv – cultivated

rather unpredictable; some clones are fertile at both diploid and tetraploid levels. Others are completely sterile at the tetraploid level. Conversely, all inter-specific AB clones are sterile at the diploid level, but showed to be systematically male and female fertile at the tetraploid level (personal observation). These results indicate that gamete sterility in AB clones is probably due to incomplete chromosome pairing at meiosis.

In 1993, chromosome number investigations were carried out on plants from intra-specific crosses between the wild 'Calcutta 4' clone, used as tester, and four doubled-diploid clones (Guyod, Galéo, IDN110 and Tjau Lagada) used as donor of polyploid gametes. The selfing of 'Calcutta 4' (tester) resulted in strictly diploid progenies (22 chromosomes), proving that all the male and female gametes of 'Calcutta 4' have 11 chromosomes. Almost all the 347 hybrids resulted from the crosses between 'Calcutta 4'' and the four doubled-diploid varieties showed to be triploid (over 98% of the hybrids) with the number of chromosomes varying from 32

to 38. In yet another study, an inter-specific cross (BB ♀ [wild type: Pisang Klutuk Wulung] × AAAA ♂ [variety: doubled IDN110]) showed all the 227 hybrids to be triploid and of BAA genomic constitution. These first results have strongly indicated that doubled-diploid clones are almost exclusively producing polyploid gametes containing, for a major part, exactly 22 chromosomes and also, in a smaller proportion, gametes ranging from 20 to 27 chromosomes. These results were to be confirmed later on a larger number of doubled genotypes.

Aforesaid results clearly proved that crossing doubled-diploid varieties with diploid clones do not lead to a very variable chromosomal constitution as one would have expected. On the contrary, the progeny is essentially triploid. These first outcomes obtained through $2x/4x$ crosses did validate the original pathways of banana improvement developed by CIRAD. An intensive programme of hybridisation combining various parents was engaged since 1993 to obtain many 'dessert' and 'cooking' triploid hybrids. The respective contributions of each species in the phenotypic expression of the progenies were better understood. It thus could be showed that the cooking versus sweet traits of the fruits were given by the *acuminata* parents, whereas *M. balbisiana* conferred a strong vegetative strength to the hybrids and that resistance to BLSD could be brought by anyone. Moreover, observations revealed that, in comparison with diploids, AAA and BAA triploids are characterised by more vigour and bunches of a better average size and weight. It was also observed that inter-family variability of progeny depends mainly on the parents selected, but that it is always much larger than intra-family variability. Lastly, while the *parthenocarpy* character segregates among the triploid progeny when *M. balbisiana* (wild type) is used as a female parent, no segregation of this character is observed in AAA progeny when the two *acuminata* parents are cultivars.

This strategy has several advantages: the genetic combinations selected at the diploid level are totally or partially preserved in the eventual triploids; the hybrids display the desired sterility because of their triploid nature; utilization of very fertile wild parents sometimes enables production of large triploid progenies in which selection is very effective (Table 1.7) and that this strategy enables breeders to tap the wide diversity in diploids. New selection criteria can also be included at any stage – by using new parents – to respond quickly either to the appearance of new races of pathogenic fungi or to meet other selection objectives.

Since 1995, CIRAD selected several good cooking as sweet BAA hybrids obtained from crosses between *M. balbisiana* clones, used as female parent, and a wide panel of *M. acuminata* autotetraploids (AAAAcv) taken as male parents. Unfortunately, because the activation of the BSV sequence integrated in the DNA of the *M. balbisiana* clones, the synthesis of BAA had to be stopped (please see section constraints). In 1996, CIRAD began to select new synthetic triploid AAA. According to parental combination, these hybrids have very different agronomic attributes (precocity and ratooning). Some of them show resistance to SD, BLSD, *Fusarium* wilt and nematodes. Fruits of these new varieties are very variable in shape, colour and are often of different tastes (more acidulated, more sweetened, etc.). They have been submitted to physiological and physicochemical characterisa-

Table 1.7 Comparison of progenitor fertility, hybrid production and selection ratios (% success) for the two crossing strategies

	Development of tetraploids $3x \times 2x \rightarrow 4x$ parent, female cultivar		Development of triploids $2x \times 4x \rightarrow 3x$ parent, female wild	
	Number	Ratio (%)	Number	Ratio (%)
Hybrid production				
Pollinated bunches	1000		1	
Bunches nearing maturity	860		1	
Bunches containing seeds	258	26	1	100
Seeds per bunch	3		3000	
Seeds (total)	774		3000	
Embryos cultured in vitro	774		500	
Embryos germinated in vitro	193	25	250	50
Individuals in the field (total)	174	22	250	50
Hybrid selection				
First selection for putative tetraploids	8	5		
			← For hybrids of plantains	
Final selection of tetraploids	2	1		
First selection of triploids			50	20
	For hybrids of Silk type →			
Final selection of triploids			15	6

tion to better define their food value, and their potential for transport, conservation and ripening conditions for national and export markets.

1.7 Varietal Screening and Utilization

1.7.1 Field Selection

Field selection of plant hybrids depends upon the objectives for selection and the overall organisation of the research institute willing to implement, as far as possible, a network of field trials combining diverse genotype/environment interactions. For banana, like in many other crops, the first selection phase is performed on highly heritable characters with a limited number of replicates and in a single location having the main pressure criteria. As the selection process goes on, new selection criteria are added and the number of replicates per genotype progressively increases. The second phase aims to select genotypes on less heritable characters and to

evaluate their behaviour in different environments. Nevertheless, as well as heredity, very little is known about heritability of the major agronomic features in banana. Thus, in the absence of this basic knowledge, selection is difficult and one should pay careful attention to avoid any mis-selection. By way of illustration, how CIRAD in Guadeloupe runs the selection process for triploid dessert export bananas is described here. The overall selection process is divided in to four main phases. It requires at least 12–15 years before a new variety is released to producers and enters production.

The first selection phase is performed on the triploid segregating progenies from the crosses between diploids and doubled-diploids. There is no replicate and each plant in the field is a unique genotype. Cavendish plantlets from in vitro culture are planted together with the hybrids as reference. Since all hybrid plantlets are borne from embryo rescue, experience has proved that the first crop cycle is biased and thus, no measurements are taken. Hybrids are evaluated in the second and the third cycle. During this phase, several hybrids showing critical drawbacks are eliminated. Attributes such as non-pendulous bunch, huge infection of Sigatoka, plant size higher than 130% of the Cavendish, bunch weight less than 50% of the reference, duration of the return, fruit size less than 14 cm, fruit diameter less than 27 mm, minimal fruit green life shorter than Cavendish and unpalatable fruits are benchmarks for selection. About 95–98% of the genotypes are usually discarded during this first phase of selection.

The second phase of selection is then directly performed for two successive cycles on five plant replicates issued from suckers taken from each plant kept after the first phase. During this phase, critical drawbacks are evaluated again. Harvesting criteria and overall plant morphology features (leaf breakage; leaf erection; suckering) are also evaluated. Preliminary consumption tests with consumer panels are also carried out. A specific testing design is used in the field to evaluate all hybrids against SD (Orjeda 1998). Every hybrid plant is surrounded by Cavendish control plants, susceptible to SD, to ensure a high and regular infestation. Observation of disease evolution and severity is noted and used as a crippling criterion at this stage.

Upon completion of this second phase, less than 5–10% of the hybrids are kept for the third phase. At this step, selected plants are introduced in vitro for rapid multiplication in order to obtain the large number of replicates required to perform the third selection phase.

For the third evaluation phase, higher quantity of plant material is required to evaluate agronomic productivity, root nematode resistance and fruit quality features (harvesting and the packaging of the fruits in industrial conditions, tolerance to cool temperatures in shipments, etc). This phase is usually performed over 3 cycles where 150–250 tissue culture issued replicates per genotype are tested. During this phase, fruits are shipped to Europe for consumer tests to evaluate the acceptability of these new bananas that strongly differentiate from the Cavendish variety. Economic studies are also carried out to evaluate the production costs.

Moreover, the third phase will implement selection criteria that cannot be evaluated in French West Indies (Guadeloupe and Martinique). For example, black leaf streak (BLS) resistance will be evaluated in controlled conditions in Montpellier,

France (Abadie et al. 2005) with various strains of the fungus showing different patterns of virulence and aggressiveness. The level of resistance will be later specified in open field conditions thanks to trials carried out in Mayotte (Indian Ocean) where the disease naturally occurs. In a similar perspective, *Fusarium* wilt resistance will be confirmed by sending plant material to international partners having the expertise to carry out such an evaluation.

After five years of evaluation, valuable hybrids will be proposed to growers for multi-plot field evaluation in different environmental conditions and different farming systems. Positive and negative points brought by growers will be carefully taken into consideration. If these problems can be solved by an agronomical or technical approach, the new hybrid will continue its procedure of validation. However, this step may represent a dead-end of the selection process for a given genotype due to unpredicted and insurmountable drawbacks. Lessons are drawn from these experiments and these new selection criteria will then be reinstated earlier in the process of selection. At the same time, a new variety has to be proposed to the growers. From CIRAD's experience, less than 2% of initial hybrid population is kept for the third phase and less than 1% will be proposed to growers for multi-field plot evaluation.

1.7.2 International Musa Testing Programme

The International Musa Testing Programme (IMTP) is a collaborative effort coordinated by INIBAP to evaluate elite *Musa* varieties in suitable sites worldwide. IMTP trials are designed to be replicated anywhere in the world. IMTP was first developed to carry out detailed evaluations of new material, in order to obtain information on their resistance/tolerance to BLSD, SD and *Fusarium* wilt. In addition, these sites may be used for studies designed to answer key questions about the pathogen, disease and host.

The establishment of the IMTP phase I began in 1989 as a programme to evaluate germplasm from the FHIA breeding programme in Honduras for resistance to BLSD. Seven tetraploid hybrids from different genetic backgrounds were tested along with several diploid reference clones (both wild and edible) that represented the whole range of reaction to BLS, from highly resistant to highly susceptible. The experiments were established in six countries. Four years later, the recommendations were made to release three clones for local consumption with good performances and high resistance to BLS: FHIA-01 and FHIA-02 as dessert banana varieties and FHIA-03 as cooking banana. Over the last 10 years, these 3 clones have been distributed to more than 50 countries worldwide.

The second phase of the IMTP started in 1996. The germplasm was evaluated for resistance to three diseases instead of one: BLS, Sigatoka and *Fusarium* wilt. Four programmes (FHIA, Honduras; EMBRAPA, Brazil; INIVIT, Cuba; and TBRI, Taiwan) contributed germplasm and the number of testing sites increased from 6–37, despite the fact that the trials were funded by the participating institutes. The results suggested that FHIA-23 and SH-3436-9 were the most tolerant to BLSD. They also

performed very well in terms of yield, especially the FHIA hybrids. The improved hybrid with the best overall performance was FHIA-23. An improved cultivar that deserves special mention is GCTCV-119 that had good yield under good management.

1.8 Allied Attributes

1.8.1 Fusarium Wilt (from Moore et al., 1995)

Fusarium wilt or Panama disease of banana is widely regarded as one of the most destructive plant diseases in recorded history. It is caused by the soil-inhabiting fungus *Fusarium oxysporum Schlecht* f. sp. *cubense* (E. F. Smith) Snyd. & Hans (Foc). The disease was first recognized in Australia in 1874. *Fusarium* wilt has now been reported from all banana growing regions of the world except Papua New Guinea, the South Pacific Islands and some of the countries bordering the Mediterranean.

Fusarium wilt is a serious problem on many banana cultivars grown by smallholders for local consumption. For example, widely grown clones in the ABB 'Bluggoe' and AAA 'Gros Michel' sub-groups are susceptible to the disease. Cavendish cultivars in subtropical countries such as Taiwan, Spain (Canary Islands), Australia and South Africa are also being increasingly attacked by *Fusarium* wilt. It is thought that plants in these areas are predisposed to systemic infection by certain strains of Foc by cold stress during winter. However, recent losses of Cavendish growing in export plantations in Malaysia, Sumatra and Java make it clear that other strains are quite capable of systemically infecting cultivars such as Valery, Grande Naine and Williams under tropical conditions.

The classical and conspicuous external symptom of *Fusarium* wilt of banana initially appears as yellowing of the leaves which then collapse at the petiole and hang down to form a 'skirt' of dead leaves around the pseudostem. A susceptible banana plant infected with Foc rarely recovers. However, poor growth of the clump may continue for some time and many infected suckers may be produced before the clump finally dies.

Infection occurs when the pathogen penetrates the roots of the banana plant. The fungus then invades the xylem vessels and, if not blocked by vascular occluding responses of the host, advances into the corm. The fungus can survive in soil for up to 30 years as chlamydospores in infested plant debris or in the roots of alternative hosts. Spread of the pathogen locally, nationally and internationally is most commonly by infected rhizomes or suckers and attached soil.

Four races of Foc are currently recognized. The term race is used less formally for this pathosystem since the genetic basis for susceptibility and resistance has not been characterised. The currently described races of Foc refer to strains of the pathogen that have been observed to be pathogenic to particular host cultivars in the field. Race 1 is pathogenic to cultivars in the AAB 'Silk' and 'Pome' sub-groups and on AAA 'Gros Michel'. Race 2 is pathogenic to ABB 'Bluggoe' and other closely related cooking bananas. Race 3 has been recorded on Heliconia

species and has little to no effect on banana Race 4 attacks AAA 'Cavendish' and all cultivars attacked by races 1 and 2. Nevertheless, it is not always possible to characterise populations of Foc using pathogenicity tests due to plant–environment interactions. Vegetative compatibility is a technique based on asexual mechanisms that occur naturally in Foc. This can be used in the laboratory to identify genetically isolated groups known as vegetative compatibility groups (VCGs) (Ploetz 1990). Strains of Foc that belong to the same VCG are genetically identical or very similar with respect to other characteristics and are, therefore, more closely related to each other than vegetatively incompatible strains. Studies have shown a strong correlation between VCG and pathogenicity in Australia, but, since with Foc more than one race can occur within a VCG and there can be more than one VCG in each race, it is often difficult to use VCG for pathotype discriminations.

Chemical control, flood fallowing, crop rotation and the use of organic amendments have not been effective in managing *Fusarium* wilt. It is now generally accepted that the only effective means of control is by host resistance. Natural sources of resistance exist in wild species and cultivars and in synthetic diploids developed by breeding programmes. Biotechnology, mutation breeding and somaclonal variations are also being used to produce resistant genotypes.

1.8.2 Sigatoka Leaf Spot Disease (from Mourichon et al., 1997)

SLSD involves two related ascomycetous fungi: *Mycosphaerella fijiensis* Morelet, which causes BLSD<end parenthetical comma>, and *M. musicola* Leach ex Mulder, which causes SD. The two pathogens can be distinguished morphologically essentially on conidia and conidiophore characteristics.

Symptoms of BLSD and SD are sometimes difficult to differentiate. In general, the first symptom is the appearance on the upper leaf surface of pale yellow streaks (SD) or dark brown streaks on the lower leaf surface (BLSD), both 1–2 mm long that enlarge to form necrotic lesions with yellow haloes and light grey centres. Lesions can coalesce and destroy large areas of leaf tissue that result in reduced yields and premature ripening of fruit. BLSD is more serious than SD because symptoms appear on younger leaves. BLSD also affects many cultivars that have resistance to SD, such as those in the plantain sub-group (AAB). Yield losses of up to 50% have been reported in some cases.

Mycosphaerella musicola was first identified in Java in 1902. Since 1962, it has been reported in most of the world's banana growing areas, making SD one of the most important epidemic plant diseases. BLSD was first recognised in Fiji in 1963. Subsequently, the disease has been reported throughout the Pacific and Asia. BLSD was identified in Honduras in 'Latin America' for the first time in 1972. It spread northwards to reach Mexico and towards Central and South America. BLSD has recently been reported in the Caribbean. In Africa, the first records were in Zambia in 1973 and in Gabon in 1978. BLSD spread along the west coast. An introduction occurred on the island of Pemba around 1987 and BLSD spread from there to Zanzibar and the coastal areas of Kenya and Tanzania. BLSD is also found

in Malawi and the Comoro Islands. BLSD and SD are disseminated locally by ascospores and conidia. Long distance spread is believed to be by the movement of germplasm (infected suckers and diseased leaves) and windborne ascospores.

Systemic fungicides provide effective control of BLSD in commercial plantations, but their effects on the environment are cause for concern. The frequency of spray applications can be appreciably reduced when used in conjunction with disease forecasting systems. However, resistance to some of these fungicides has been developed by both *M. fijiensis* and *M. musicola* strains in the Caribbean, Central America and Africa. Genetic resistance to BLSD and SD is clearly the best long-term goal for disease control especially for smallholders who cannot afford to purchase chemicals.

The inheritance of the BLSD resistance from the wild diploid Calcutta 4 has been studied in various crosses. Progenies of crosses made between wild diploids (AA) are now being studied in order to understand the inheritance of BLSD resistance and to localize genes or quantitative trait loci (QTLs) using genetic maps (Carreel et al. 1999).

1.8.3 Nematodes

Worldwide, the nematode species known to cause the most serious damage to bananas *sensu lato* are the migratory endoparasites, *Radopholus similis*, *Pratylenchus coffeae* and *Pratylenchus goodeyi*, the endoparasite *Helicotylenchus multicinctus* and the sedentary parasite *Meloidogyne* spp. In addition to these five major species, many other species have been reported to be associated with *Musa* spp. throughout the world. Depending on local conditions, the associated damages of any of these nematode species may be locally important where their densities are high (Gowen et al. 2005). Migratory endoparasites cause similar root and corm damages. The migratory feeding behaviour of these nematodes in the root and corm tissues causes the formation of discoloured lesions, which may enlarge and coalesce, resulting in large necrotic areas.

Nematodes infection can be controlled by nematicides in the field or by modified agricultural practices (planting of healthy banana tissue cultured plants in cleaned soils) (Chabrier and Quénéhervé 2003). Thus, nematode resistance is not yet the priority for dessert banana breeders in comparison to *Fusarium* wilt or SLSDs. In addition, resistance to nematodes through genetic improvement is hindered by difficulties associated with banana breeding. Despite these difficulties, some progress has been made. Several clones of ‘Pisang Jari Buaya’ (AA) have been recognized as an exploitable source of resistance to burrowing nematode. Although its inheritance has not yet been established, the resistance of Pisang Jari Buaya has been incorporated into breeding lines by FHIA and has led to the production of hybrids of commercial interest. At CIRAD, some AA cultivated clones have also been identified as tolerant or resistant to several species of nematodes (Quénéhervé et al. 2006) and most of their triploid progenies shown to be more tolerant to these pathogens than the Cavendish varieties of reference (Quénéhervé, pers. com.).

1.8.4 Drought and Cold Tolerance (from Israeli and Lahav, 2000)

The banana tree is a tropical plant originating in the wetlands. Thus, optimal growth conditions require no water stress, moisture of the air and temperatures ranging from 28°c to 36°c. In subtropical conditions, banana cropping is subject to water shortage and cold temperature that give rise to growth and quality disorders. For instance, in the Mediterranean producing countries, intensive Cavendish production is shown as a high water consuming crop, irrigation being one of the main production problems. In some countries such as Morocco and Turkey, cold temperatures during winter are considered as the main agronomic constraint. Therefore, banana production requires, in these conditions, huge irrigated greenhouse infrastructures to reduce day/night temperature fluctuations and to avoid frost damages during the coldest nights.

The banana genetic diversity is much reduced in the Mediterranean region and would certainly deserve to be increased by introducing new germplasm. On the other hand, sensitivity to drought and frost varies among cultivars, varieties containing the B genome being more resistant to abiotic stress than those solely based on the A genome. For instance, in Egypt where banana genetic diversity is higher than the rest of the region, the traditional AAB and ABB varieties cultivated in rural areas proved to be more resistant or tolerant to drought than the Cavendish ones (De Langhe 2002). Another is the 'Sugar' ABB Pisang Awak variety grown in Oman where it is shown to be well adapted to dryness at the Agriculture research station of Salalah (De Langhe 2002). There are very few ABB dessert varieties showing good palatability and high productivity in the natural germplasm. Therefore, the triploid breeding strategy offers good future prospects through the combination of edible AB cultivars with wild *balbisiana* to create new productive dessert ABB varieties, palatable and tolerant to drought and cold temperatures.

1.9 Biotechnology

Remarkable progress has been made during the last two decades on micro-propagation of bananas through proliferation of vegetative meristems in vitro. This method is now widely used to promote the exchange of germplasm and to produce material for planting. Many morphological and agronomic variations have appeared in plants obtained by micro-propagation (Vuylsteke et al. 1991; Côte et al. 1993). Hence, given the difficulties in conventional breeding, several research groups have attempted to increase the variability of bananas in the Cavendish sub-group by focusing on somaclonal variations (Daniells and Smith 1993) or inducing mutations artificially (Roux 2004). Biotechnological tools are presently used routinely to enhance the effectiveness of conventional breeding programmes.

1.9.1 Somaclonal Variations

Although carrying out recurrent selection over several successive cycles, each including a micro-propagation phase and a selection phase in the field, Cavendish

clones were identified with new resistance (to pathogens/pests), fruit quality and productivity traits. Among the in vitro plants of a traditional Cavendish variety, susceptible to tropical race 4 of *Fusarium* wilt, a variant clone resistant to the disease, Pei-Chiao, was selected by TBRI in Taiwan. This clone had unfavourable agronomic characteristics but, after in vitro multiplication, led to another clone, Tai-Chiao No.1, with resistance to the disease and improved production characteristics (Tang and Hwang 1994).

1.9.2 Experimental Mutagenesis

To widen the range of genetic variations in Cavendish, other research groups have worked to induce artificial mutations by chemical or physical treatments – gamma rays in doses of 20 to 60 Grays. Generally, mutagenic treatments are applied on proliferating buds cultured in vitro. The regenerated plants have a number of modifications in height, leaf shape, shoot growth and bunch features (Novak et al. 1990).

The IAEA selected an early maturing clone of Grande Naine, GN-60A, in which the bunches were more cylindrical and large with superior organoleptic qualities. From the Cavendish variety Extra Naine, Dwarf Parfitt (known for its natural resistance to the tropical race 4 of *Fusarium* wilt, but of no commercial value) was developed in Australia. The QDPI (Australia) created a very large mutant, Giant Parfitt, which inherited this resistance and has acceptable agronomic and commercial value.

1.9.3 Zygotic Embryo Culture

Banana seeds resulting from manual pollinations are often malformed and sometimes immature. Planted in a nursery, their germination rate is very low (i.e. from 0% to around 25% according to the crosses). Embryo rescue in vitro has significantly increased this germination level (as much as 95%) under optimal conditions (Table 1.7). In banana breeding programmes, this technique is now currently used to increase the size of the progenies and to give access to the evaluation of plant material coming from new parental combinations for which seeds do not germinate in traditional sowing (Bakry and Horry 1992a).

1.9.4 Somatic Embryogenesis

Somatic embryogenesis focuses essentially on two objectives: development of new effective micro-propagation techniques and of cell regeneration systems necessary for the development of non-conventional breeding programmes. The technique was successfully applied to diploid immature embryos and then to plant tissues (Novak et al. 1989; Dhed'a et al. 1991) and floral tissues (Escalant et al. 1994). Regeneration of plants by adventitious embryogenesis and cell suspensions has also progressed

(Côte et al. 1996). These embryogenesis methods were developed in very different genotypes: cultivars of dessert and cooking triploid bananas of agronomic interest, as well as diploid clones useful for genetic improvement programmes.

1.9.5 Cryopreservation

Cryopreservation can be practiced using embryogenic cell suspensions (Côte et al. 2000; Panis et al. 2004) and proliferating meristems (Panis et al. 2005). It could be also applied for long-term conservation of banana seeds (Bhat et al. 1994). This technique avoids micro-propagation, which involves much labor for large collections and may also cause mutations as well as loss of accessions due to contaminations or human error (Panis et al. 1990). Associated with somatic embryogenesis, cryopreservation also enables storage of industrially produced in vitro plants in the form of frozen cell suspensions. The quality and conformity of the stored material can also be checked: an agronomic evaluation may be carried out on a plant sample after thawing and regeneration (Côte et al. 2000). More recently, cryopreservation in banana has also been shown as a powerful tool for the sanitation of varieties infected by viruses (Helliot et al. 2002).

1.9.6 Haploid Methods

With the exception of some wild bananas, all diploids used in hybridisation are heterozygous that varies from 20 to 70%. They also have structural heterozygosity namely, translocations and inversions enforcing sterility in diploids thus leading to a high degree of variability among gametes. Breeding programmes could be more effective if more fertile lines were available.

The first androgenic banana plants were obtained from the wild clone Long Tavoy of *M. acuminata* (Bakry and Horry 1992b). Factors influencing the induction of androgenic calluses – stages of anther excision, culture media – and the regeneration of whole plants were later specified by Kerbellec (1996). To date, numerous androgenic calluses and several haploid and diploid plants have been produced from various *M. acuminata* and *M. balbisiana* clones (Assani et al. 2003).

1.9.7 Protoplast Culture and Somatic Fusions

Whole plants (of CV Bluggoe – ABB) have been regenerated from protoplasts isolated from embryogenic cell suspensions and cultured on feeder layers of Lolium (Megia et al. 1993). The plants were evaluated in the field in Guadeloupe to determine the influence of ‘protoplast’ stage on somaclonal variations. Based on these early results, a research team from the University of Paris (France) began work on somatic fusion that should provide valuable support for conventional breeding

programmes (Assani et al. 2002). The technique can accelerate or even facilitate crossing that is difficult or even impossible to achieve by classic methods. This initial phase involved somatic hybridisation between diploid bananas to develop tetraploid elite parents and then back-crossed to produce triploids (Haïcour et al. 2004). The development of haploid methods should then enable fusion of haploid and diploid protoplasts, leading to the direct production of novel triploid varieties.

1.9.8 Genetic Transformation

Several research centres were early involved in this work, including the KUL, Belgium, BTI associated with Cornell University (USA), CATIE (Costa Rica), IAEA (Austria), QUT (Australia) and CIRAD (France). Transgenic banana plants were first obtained by particle bombardment of cell suspensions (Ságі et al. 1995) and by *Agrobacterium tumefaciens* inoculated on in vitro sections of plant meristems (May et al. 1995) and on cell suspensions (Khanna et al. 2004).

Priority is given to transferring genes to confer resistance to viruses – CMV and BBTV – as there are no varieties naturally resistant to these pathogens. Moreover, strategies for the acquisition of these resistances by genetic transformation are now available. Considerable damage inflicted by weevils and nematodes justifies rapid implementation of research programmes focusing on protease inhibitors and *Bacillus thuringiensis* (Bt) genes. The damage caused by *Mycosphaerella* and *Fusarium* fungi has already prompted research to identify resistant genes – chitinases, antifungal proteins and so forth (Pei et al. 2005). Several groups have also considered modifying the metabolic routes that control ripening – the anti-sense gene 1-aminocylopropane-1-carboxylate synthase – to stop ethylene synthesis and to decrease the maturation of the fruits in non-refreshed post-harvest conditions. Recently, researchers from the Bhabha Atomic Research Centre, India, investigated the possibility of producing hepatitis B antigen in banana with the aim of developing an oral vaccine (Kumar et al. 2005). This procedure now needs to be validated. It also raises bioethical questions.

1.9.9 Molecular Markers and Genomics

RFLP, nuclear and cytoplasmic molecular markers have been first applied to manage genetic resources in banana. Mapping of genetic diversity by PCR was later developed to enable genetic diversity studies and genetic mapping using non-radioactive technologies.

These studies enabled characterisation of genetic variability, determination of the extent of heterozygosity and characterisation of phylogenetic relationships between wild, diploid and triploid clones (Carreel et al. 1994). These results are now being used by CIRAD to define cross strategies leading to better parental combinations (Raboin et al. 2005).

The first genetic map of banana was established on 90 loci-58 RFLP, 4 isozymic and 28 RAPD markers. Among these loci, 77 could be partitioned into 15 linkage groups and 13 independent loci. This map was completed with 30 micro-satellite markers. A second map was made: it comprised more than 300 markers, distributed in 11 linkage groups, which correspond to the basic genomic number in bananas (Noyer et al. 1997). By combining these two maps, it was possible to come up with an outline map based on 130 locus-specific markers. This map could be helpful in carrying out marker-assisted selection (Carreel et al. 1999).

1.9.10 Genomics

Developing basic genomic tools to assist germplasm exploitation is important for banana, especially in the context of the use of *Musa* genomic diversity. Genomic studies in banana are currently developed within the framework of the Global *Musa* Genomics Consortium, an international network of investigators, for establishing *Musa* as a model crop for studies on comparative genomics leading eventually to sequencing of the banana genome (INIBAP 2005). In comparative genomics, *Musa* is seen as an ideal model for understanding genomic evolution in relation to biotic and abiotic stresses, in a polyploid, vegetatively propagated crop. The consortium currently brings together expertise from 32 institutions in 22 countries. Consortium members have so far developed BAC libraries from *M. acuminata* and *M. balbisiana* (Vilarinhos et al. 2003; Safar et al. 2004). The first sequencing of some BAC clones showed that less than 50% of the DNA in these clones was coding for genes. Like most plant genomes, the banana genome seems to comprise gene-rich areas that are separated by long stretches of repetitive sequences (INIBAP 2005). More recently, the sequencing of several *acuminata* and *balbisiana* BAC clones led to the identification of resistance gene analogs (RGAs) and genes coding proteins involved in abiotic stresses (salt tolerance, low temperature, heat shocks and drought tolerance). In addition, as scientists have known for some time, the B genome – and maybe the A genome as well – contains viral DNA from the BSV, which, in the course of the evolution of *balbisiana*, has found a niche in the banana's genome.

1.10 Conclusion

The genetic basis of the varieties cultivated in the world is extremely narrow and the risk of disappearance of this culture due to the emergence of new diseases is high. As of today, resistance to *Fusarium* wilt and SLSDs is a priority. New strains of *Fusarium* wilt recently emerged in Australia, South Africa and some Asian regions and are infectious on Cavendish varieties. It is essential to breed resistant varieties for banana production and export. In Central America, chemical control of BLSD becomes lesser effective and requires the application of an increasing quantity of fungicides. Moreover, the pathogen circumvented the resistance of some natural

varieties especially in the Pacific region. The challenge for the future is to perpetuate banana production while preserving environment.

Export markets in North America, Europe and Japan mainly deal with single variety (Cavendish). Great efforts have been incurred in the recent past to promote banana markets through the differentiation of farming system. Banana breeding is also thought of as a means to sustain diversification allowing a fruit segmentation on the export markets.

Diploid varieties can cumulate various sources of resistance to diseases and also genes encoding for good fruit quality. In this perspective, marker-assisted selection will be an invaluable tool for the improvement of diploids within *M. acuminata* as well as to seek and select new clones of *M. balbisiana* free from integrated sequences of badnavirus.

A better understanding and exploitation of combining abilities between clones at the triploid level is desirable. In particular, research should explore new genetic combinations (having recourse to specific tools such as protoplast fusion) and investigate the effects of allelic dosages in hybrids derived from interploid reciprocal crosses.

Induced mutation and genetic transformation are very useful for banana improvement. By increasing phenotypic diversity, these techniques can result in plant types with reduction in plant size and improved bunch and fruit shapes.

Conventional breeding through hybridisation has been the way by which almost all cultivated plants have historically been improved. As stated by Rowe and Rosales (2000), banana was recalcitrant in this regard, but it has now joined the long list of crops that are improved by cross-pollinations and selection. New breeding strategies offer good opportunities to create varieties well adapted to the various improvement objectives for local consumption as well as for export markets.

Research Institute Acronyms

BRS: Banana Research Station, Kerala Agricultural University, India.
BTI: Boyce Thompson Institute for Plant Research, USA.
CARBAP: The African Centre for Research on Banana and Plantain, Cameroon.
CATIE: Centro Agronómico Tropical de Investigación y Enseñanza, Costa-Rica.
CIRAD: French Agricultural Research Centre for International Development, France.
EMBRAPA-CNPMF: Empresa Brasileira de Pesquisa Agropecuária Centro Nacional de Pesquisa de Mandioca e Fruticultura Tropical, Brazil.
FHIA: Fundación Hondureña de Investigación Agrícola, Honduras.
IAEA: International Atomic Energy Agency, Austria.
IITA: International Institute of Tropical Agriculture, Nigeria.
KUL: Katholieke Universiteit Leuven, Belgium.

NRCB: National Research Centre for Banana, India.
QDPI: Queensland Department of Primary Industry, Australia.
QUT: Queensland University of Technology, Australia.
TBRI: Taiwan Banana Research Institute, Taiwan.
TNAU: Tamil Nadu Agricultural University, India.

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Chapter 2
Mango Breeding

Ian S.E. Bally, Ping Lu, and Peter R. Johnson

2.1 Introduction

The common mango (*Mangifera indica* L.) and closely related genera (*Mangifera* spp.) belong to the family Anacardiaceae that consists of dicotyledonous trees and shrubs. Mango trees are evergreen with branched, upright to spreading dense canopies that can grow to heights of 30 m. The tree is supported by one to several deep taproots and abundant surface feeder roots. Trees are long lived with many specimens living for more than 100 years. The canopy consists of dark green, simple, alternate leaves, oval-lanceolate to roundish-oblong in shape. New leaves are produced in periodic flushes with the colour tone of expanding leaves varying between tan and red. Hundreds of hermaphrodite and male flowers are borne on branched conical panicles that grow from the terminals of branches. The fruits are fleshy drupes that vary in size, shape and colour, with the fleshy mesocarp being the most attractive edible proton of the fruit. Each fruit contains a single seed enclosed in a stony endocarp. Seed embryos can be either monoembryonic or polyembryonic dependant on genotype.

Mango by production is the fifth largest fruit crop in the world behind bananas, grapes, apples and oranges and the second most important tropical fruit crop. Global production is estimated to be around 27 million tonnes per annum. India dominates global production accounting for some 40% of total production. Mango production plays an important role in the rural economy of many tropical countries. However, in spite of the large volume of mango production almost all of the product is consumed domestically in its country of origin: less than 4% (908,000 tonnes) of production is exported. Many of the reasons for the low export volumes lie in the highly perishable nature of mangoes. Of the mangoes that are grown and traded internationally, the majority are cultivars originating in Florida which are renowned for their strong blush colour and longer shelf life. Cultivars such as 'Tommy Atkins', 'Kent' and 'Keitt' dominate global trade. Markets with close proximity to the country of origin

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have enabled export markets to develop based on more perishable cultivars such as ‘Carabao’ from the Philippines and ‘Sindri’ from Pakistan. Recent developments in Plant Breeders rights (PBR) have seen the exclusive commercial development of many new mango varieties and a renewed interest in breeding and selection programmes.

2.2 Genetic Resources

2.2.1 Origin of Mango and Genetic Resources

Mangifera species are found growing throughout the tropical and subtropical world today, with the greatest diversity of species in the Southeast Asian regions of the Malay peninsula, the Indonesian archipelago, Thailand, Indochina and the Philippines (Bompard 1989; Bompard and Schnell 1997). The most commonly cultivated species is *M. indica* that makes up most of the commercially cultivated trees. Twenty-six other species also have edible fruit with *M. caesia, M. foetida, M. kemang, M. altisima, M. panjang, M. odorata, M. laurina* and *M. pentandra* being traditionally consumed in various Southeast Asian communities (Gruezo 1992; Bompard and Schnell 1997; Mukherjee 1997).

The common mango (*M. indica*) is thought to have originated in the Assam valley in Myanmar and domesticated independently in several areas to the southwest and southeast of the centre of origin. Domestication in the Indian region (southwest) gave rise to the monoembryonic varieties and domestication in the Indochina, Thailand and Myanmar regions (southeast) gave rise to the polyembryonic varieties (Bompard and Schnell 1997). Cultivation of monoembryonic mangoes in India for over 4,000 years has resulted in over 1,000 named varieties (Mukherjee 1953). Domestication and discrimination of the common mango throughout the tropical and subtropical world has been associated with the migration of people and trade within and between regions (Mukherjee et al. 1983; Bompard and Schnell 1997; Duval et al. 2006). Today mangoes are grown throughout the tropics on all continents and in many subtropical areas.

Many of the current commercial varieties grown in the world today originated from a secondary centre of diversity in Florida during the twentieth century. From 1861, many varieties of mangoes from different regions of the world were introduced to Florida, resulting in a wide diversity of mango germplasm in the area (Knight 1980). This diversity and mangoes’ tendency for natural out-crossing led to the production of hybrid seedlings with novel characteristics. In 1910, a high yielding monoembryonic seedling of the variety ‘Mulgoba’ with an attractive red blush was selected in Florida and named ‘Haden’. During the twentieth century, other seedling progeny selection were made from ‘Mulgoba’, ‘Haden’ and other introduced cultivars, such as ‘Keitt’, ‘Kent’, ‘Tommy Atkins’, ‘Glenn’, ‘Lippens’, ‘Van Dyke’, ‘Parvin’, ‘Springfels’ and ‘Zill’ (Campbell 1992; Knight and Schnell 1994). The success of the Florida varieties is due to their adaptability to many agroclimatic regions of the world while retaining their fruit quality and regular

bearing characteristics. Detailed descriptions of mango varieties from Florida have been published by Campbell (1973), Campbell and Mallo (1976), Campbell (1992), Campbell and Campbell (1993) and Knight and Schnell (1993).

2.2.2 Varieties and Genetic Resources

Although a relative few varieties are considered good commercial cultivars suited to national and international trade there are many other local minor varieties with interesting characteristics that form a vast genetic resource to the mango breeder. Local selected and wild varieties are often well adapted to local environmental conditions and provide a useful source of locally adapted genes. There have been considerable efforts in the past to collect and document mango varieties in many countries. Lists of mango collections and some descriptions of accessions in collections are available through the International Plant Genetic Resource institute website (http://www.ipgri.cgiar.org/germplasm/dbintro.htm) and in publications of Yadav and Rajan (1993), Yadav (1997).

2.3 Reproductive Biology

2.3.1 Cytology

Most *Mangifera* species are reported to be diploid with chromosome numbers of $2n = 40$ and $n = 20$ (Mukherjee 1950, 1963; Kostermans and Bompard 1993; Iyer and Degani 1997), although there is some suggestion that mango evolved through amphidiploidy (Mukherjee 1950; Mathews and Litz 1992; Yonemori et al. 2002). The high variability in leaf width and thickness and other morphological traits between varieties of *M. indica* supports a polyploidy and hybrid origin (Singh 1960). Recently, two spontaneous tetraploid mango seedlings were identified. In the Canary island, Spain, a putative tetraploid 'Gomera-1' was confirmed using both flow cytometry and chromosome count analyses (Galan Sauco et al. 2001). In Katherine, Australia, another tetraploid common mango was confirmed by chromosome count (K. Rayner and H. Stace, unpublished). Currently both these tetraploid mangoes are used for rootstock breeding purpose. The similarity of ploidy between the *Mangifera* species indicates that inter-specific crossing should be feasible. Several authors have expressed the value and contribution that inter-specific crosses between mango and many wild *Mangifera* species can make to the improvement of cultivated varieties (Mukherjee 1963; Bompard 1993; Iyer and Degani 1997).

2.3.2 Floral Biology

The mango inflorescence is primarily terminal. On a panicle the total number of flowers may vary from 1,000 to 6,000 depending on the cultivar (Mukherjee 1953).

Flowers are usually small, 5–10 mm in diameter. Both perfect (hermaphrodite) and male flowers (Fig. 2.1) occur on the same panicle. The ratio of perfect to male flowers varies with cultivar (usually < 50%) but is strongly influenced by environmental and cultural factors (e.g. cool temperatures usually reduce the number of perfect flowers, which is more pronounced in tropically evolved polyembryonic cultivars) (Davenport and Nunez-Elisea 1997). Anthesis starts early in the morning and generally completes by noon. Stigma receptivity remains for about 72 h, but most receptive during the first 6 h. Minimum pollen germination time is 1.5 h (Spencer and Kennard 1955). Singh and Singh (1952) observed 98% pollen viability after 11 months in storage at 7 °C and 25% RH, and 65% viability after 24 months of storage at 0 °C and 25% RH.

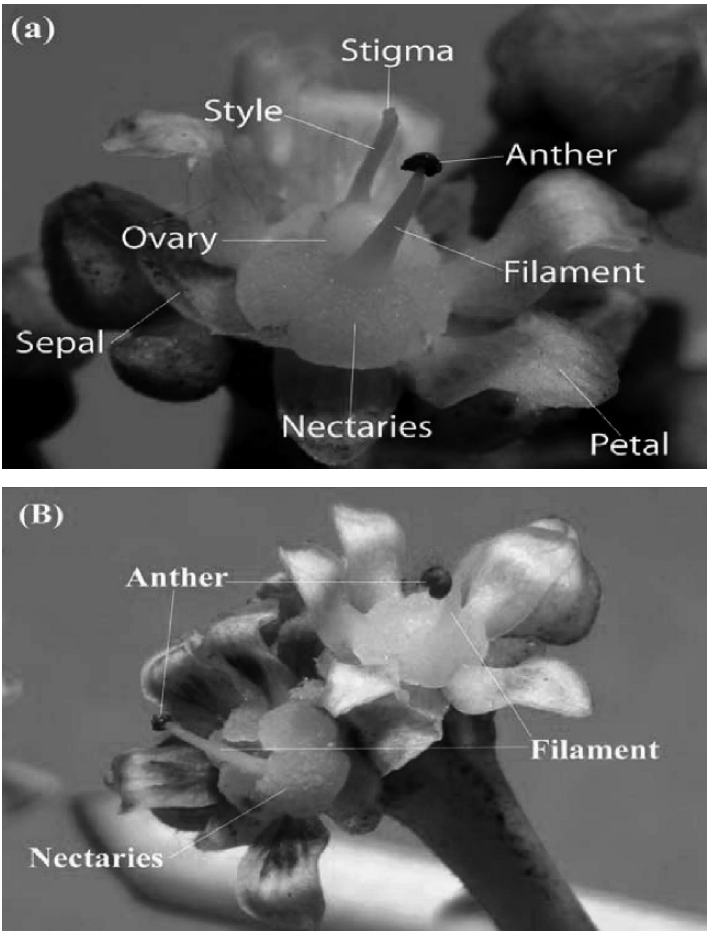

Fig. 2.1 Organs of (**a**) hermaphrodite and (**b**) male mango flower (*See* Color Insert)

2.3.3 Pollination and Compatibility

Mango is generally pollinated by insects, but pollination by wind and gravity cannot be excluded (Maheshwari 1934). The percentage of fruit set in a mango panicle is less than 1% (Usman et al. 2001) and is dependant on the pollination compatibility of the cultivars, the number of hermaphrodite flowers, pollen viability and the environmental conditions during pollination. Pollination self-incompatibility has been demonstrated in the cultivars 'Dashehari', 'Langra', 'Chausa' and 'Bombay Green' (Singh et al. 1962; Mukherjee et al. 1968; Sharma and Singh 1970; Desai and Bhandwalkar 1995). However, the situation with 'Langra' is not clear with Narayana Swamy et al. (1989) finding it to be self-fertile. There are also many instances of differential fruit set and compatibility reported between cultivars. Bally et al. (2000) reported varying success rates of hybridisation between different parental combinations due to a mixture of genetic and environmental and technical factors. They were unable to produce any hybrids from controlled crossing of the cultivars 'Edward', 'Kensington Mono' and 'Magovar' as maternal parents with 'Kensington Pride'. However, the bunch bearing cultivars 'Creeping' and 'Willard' were more successful with 100% of crossed panicles bearing one or more hybrid fruit. Robbetse et al. (1994) suggested that there were clear indications of differences in receptivity existing among the cultivars, with the time of pollination and female parent receptivity playing an important role in pollination success. Robbetse et al.(1994) found the pollination success rate of 'Tommy Atkins', 'Sensation' and 'Kent' was much higher in the morning than in the afternoon, while in 'Isis' and 'Keitt' the reverse was observed.

The source of pollen in out-crossing has also been shown to have an impact on the percentage of fruit set. 'Sensation' was shown to be the best pollinator amongst the Florida cultivars (Robbertse et al. 1993). Desai and Bhandwalkar (1995) showed that 'Kesar' pollen increased the percentage of fruit set in 'Alphonso' and 'Vanraj' but reduced it in 'Totapuri' and 'Baramasi' compared with selfing. The many factors impacting on pollination make it difficult to predict the pollination success of any particular parental combination.

2.3.4 Polyembryony

Mangoes are classified as either monoembryonic or polyembryonic based on the numbers of embryos in the seed (Fig. 2.2). Monoembryonic cultivars have a single zygotic embryo whereas polyembryonic cultivars have multiple embryos one of which can be zygotic (usually a weak embryo) and the rest are nucellar in origin. The multiple nucellar embryos develop adventitiously from nucellar tissues surrounding the embryo and, as a result, are genetically similar to the tree bearing the seed (Juliano 1937). Polyembryonic seeds germinate as multiple separate seedlings, most of which are nucellar in origin and true to type. Polyembryony is genetically inherited and thought to be influenced by a single dominant gene (Aron

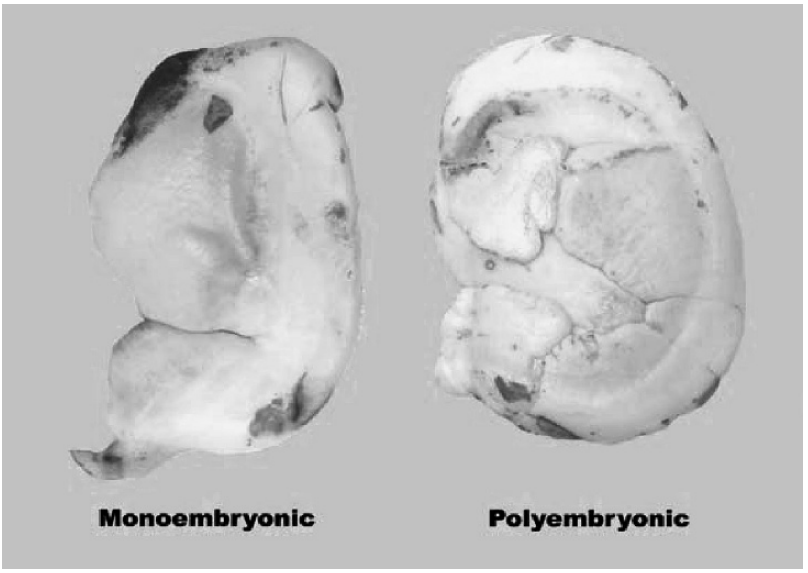

Fig. 2.2 Embryos of monoembryonic and polyembryonic mango seeds

et al. 1998; Brettell et al. 2004). However, there are some suggestions that high temperatures during flowering and fruit development cause some monoembryonic cultivars to produce polyembryonic seed (Mathews and Litz 1992; Brettell et al. 2004). The cultivar R2E2 produces predominantly polyembryonic seed with up to 30% monoembryonic seed in some seasons (Bally 1998).

Using polyembryonic cultivars for maternal parents in a hybridisation programme is problematic, as identification and recovery of the zygotic embryo is technically difficult and only possible by molecular screening techniques (Schnell and Knight 1992; Degain et al. 1993). For this reason, most open and closed hybridisation breeding programmes only use monoembryonic maternal parents.

2.3.5 Juvenility

Juvenility is the time taken for a seedling to reach a fruit bearing stage. In mangoes, juvenility is long and varies between cultivars. Mango seedlings normally take 3–10 years to bear fruit, making for a very long breeding programme (Iyer and Degani 1997). Although the long juvenility can often be overcome by grafting on to mature trees (Singh 1957), girdling of young seedlings or by the application of paclobutrazol or ethephon (Chacko et al. 1974; Iyer and Degani 1997), these methods can also bias the evaluation of the hybrid's fruit quality attributes, such as fruit size, colour and flavour.

2.3.6 Natural Fruit Shedding

Mangoes bear multiple flowers on terminally produced inflorescences of panicles. Each panicle is capable of setting between a few to over 500 fruits, most of which are progressively shed between fruit set and fruit maturity to leave one or several fruits per panicle at maturity. In a controlled crossing situation, natural fruit shedding causes many of the hand pollinated flowers and fruits to be lost before maturity, making the percentage of successful hand crosses very low. The effects of natural fruit shedding in controlled crossing programmes can be partly overcome by crossing fewer numbers of flowers on many panicles rather than crossing many flowers on fewer panicles.

2.4 Genetics

2.4.1 Heritability of Genetic Traits in Mango

In mango there have been relatively few studies on the genetic inheritance of traits because of the difficulties in generating suitable populations for the purpose. In open pollinated populations, the pollen parent is not easily identified. The high heterozygosity and small size of populations have also limited the suitable analyses. However, a few studies have provided some guidelines for breeders.

A study by Sharma and Majumdar (1988) of various hybrid populations made over 25 years indicated that recessive genes control traits such as dwarfing and regularity of bearing, while additive genes control traits such as fruit size and skin colour. Lavi et al. (1998) studied correlations between genetic and phenotypic traits in an open pollinated population of mainly Floridian cultivars and found that the non-additive genetic variance was a major and significant component of total genetic variance and additive genetic variance was non-significant. They found the progeny performance was not easily predicted from parental performance and recommended a widening of the genetic background of the parents in breeding programmes. However, in their later work Brettell et al. (2004) demonstrated traits such as fruit weight, shape, flavour and colour were highly heritable, giving confidence in systematic selection for these traits. The high heterozygosity and lack of information on heritability on many traits are a challenge for mango breeders. The heritabilities of many other traits are discussed in Section 2.7.

2.5 Breeding Principles

Mango, like many other tropical fruit crops, is relatively underdeveloped with most improvements coming from natural selection over many generations. However, with modern consumer and market demands on the fruit, the traditional varieties are not always able to successfully compete. Systematic breeding programmes are needed

to improve the productivity and fruit quality of mango varieties to enable them to compete in markets today.

Improvement of mango through breeding has traditionally been slow and challenging, with breeding programmes often taking more than 20 years. There are a number of characteristics that contribute to the slowness and small size of conventional breeding programmes in mango, such as long generation times, long juvenile stage, high heterozygosity, polyembryony, high natural fruit shedding and cultivar incompatibility. Management of these aspects in mango breeding programmes is covered in more detail later in this chapter. Despite these difficulties, the wide genetic pool available in mango and the ability to vegetatively propagate improved selections at any stage of the breeding process (Iyer and Degani 1997) allows highly heterozygous progeny to be released as cultivars and genetic gain to be captured.

2.6 Breeding Programme Stages and Design

Most mango breeding programmes can be divided into several stages such as progeny generation, primary evaluation, secondary evaluation, regional evaluation, new cultivar release and post-release support. Each of these stages can take several to many years, and efficiency at each phase is necessary to minimise the time taken to achieve genetic gain.

2.6.1 Parental Selection

Parental selection is primarily dependant on the traits desired in the progeny and is best guided by the phenotypic expression of potential parents, but with the general poor understanding of trait heritabilities in mango and their heterozygotic nature, prediction of progeny performance based on parental phenotypes is not always possible. However, analysis of current breeding populations for trait heritabilities is likely to produce improved knowledge on inheritance of specific traits in specific families. Polyembryonic cultivars are not suitable as maternal parents as identification and recovery of the zygotic embryo amongst the nucellar embryos in the hybrid seed is not easily achieved without molecular identification and embryo recovery techniques (Degain et al. 1993).

2.6.2 Hybridisation

There are several methods that can be used to generate breeding populations in mango. These are covered in more detail in Section 2.8.

2.6.2.1 Preliminary Hybrid Evaluation

Early selection of hybrids during the preliminary evaluation phase is essential to allow early setup of the secondary evaluation trials, even if additional years of evaluation are needed for confirmation of the selections and to reduce the overall time of the breeding programme. Preliminary evaluation is usually done on a single tree basis to reduce the land, management and time resources required to grow large hybrid populations. Early selection can be achieved using traits that lend themselves to pre-selection, that is traits that can be evaluated during the juvenile stage. Examples of pre-selectable traits are disease tolerance based on leaf assays or fruit flavour based on the leaf volatiles (Majumder et al. 1972; Whiley et al. 1993). Another form of pre-selection is marker-assisted selection (MAS) that uses molecular markers linked to phenotypes or genes of interest. Although there are currently only a few developed molecular markers available for mango breeders, research and development of markers for traits of interest in mango is being conducted in several countries and the availability of molecular markers is expected to increase and provide many more pre-selection tools for breeders (Lavi et al. 2004).

2.6.2.2 Secondary/Regional Evaluation

Hybrids selected in the preliminary evaluation phase generally undergo a secondary evaluation phase to comparatively evaluate the hybrid performance with industry standard cultivars, parents and other hybrids. Secondary evaluations usually are done using multiple clonally propagated trees in several locations to obtain information on hybrid performance over a range of environments and seasons. Yield and productivity performance that are not easily measured on single trees in the preliminary evaluation phase are more reliable in secondary evaluation trials. The secondary evaluation trials are usually the first source of larger quantities of fruit for market testing and post-harvest evaluation of fruit.

2.6.2.3 Cultivar Release and Post-release Support

The information from the evaluation phases will identify hybrids with suitable traits for release as new cultivars. There are many decisions to be made when releasing a new cultivar and the process will not be covered in this chapter. The commercial release of a new variety can be slow especially if plant variety rights are involved. Early planning of release strategies will save much time at the time of release. The release and successful uptake of a new cultivar is dependant on the comparative performance with existing cultivars, the availability of planting and propagation material and specific management information on the new cultivar. Many of the large scale production issues of a new cultivar that are not apparent at the time of release can be managed post-release with a planned R&D support programme for the new cultivar. The successful release of a new cultivar will necessitate the movement of plant material between growing regions. Production of clean bud-wood or trees in

a region with minimal quarantine restrictions will make dissemination of the new variety easier. It is inevitable that any new cultivar will require different management inputs than older cultivars. Determining the specific management requirements of a new variety ahead of release and providing R&D support after release will facilitate uptake by growers and the ultimate success of the cultivar.

2.7 Breeding Objectives

The objectives and aims of mango breeding programmes will vary from region to region depending on market requirements and production constraints. In the cooler subtropical regions regularity of production is often an issue whereas in the warmer tropical regions poor flowering and low yields are an issue (Iyer and Dinesh 1997). It is important to take a market approach when setting breeding goals because without market acceptance new cultivars cannot be considered successful. Future mango cultivars will need a set of traits that provide efficient, regular production and robust fruit quality at the consumer level. Some of the more desirable traits common to most mango breeding programmes are: regular bearing, dwarf trees, attractive colour, size, shape and flesh quality, resistance to pests, diseases and disorders, extended storage and shelf life, extended production period and tolerance of adverse soil conditions (Iyer and Degani 1997; Lespinasse and Bakry 1998). The major desirable production and fruit quality traits are discussed below.

2.7.1 Juvenility

In several tree fruits, short juvenility has been positively associated with high productivity. When Lavi et al. (1989) studied the heritability of juvenility, they found the female parent did not contribute to the distribution of the juvenility period in hybrid progeny. Iyer and Degani (1997) suggested overcoming the delays caused by long juvenility in breeding programmes by pre-selection of seedlings to avoid the need of maintaining large numbers of seedlings for long periods. Traits such as leaf volatiles can be used to predict fruit flavour and xylem to phloem ratios of less than one or high phenolics in the apical bud can be used to predict dwarfing (Iyer 1991; Kurian and Iyer 1992).

2.7.2 Productivity

Yields in mango are generally low compared with other fruit crops and characterised by high seasonal variability. High, seasonally uniform yielding cultivars are needed to improve farm productivity and provide even market supply. The reasons for low and irregular productivity of mango are many. They include poor flowering and low photosynthetic efficiency during flowering and fruiting periods and the failure to

initiate new vegetative growth after flowering in late season cultivars (Chacko and Kohli 1984; Iyer and Degani 1997; Gonzalez and Blaikie 2003). Improvement in any of these traits is likely to contribute to a cultivar with improved regularity of production.

2.7.3 High Pack-Out

Pack-out refers to the percentage of harvested fruit that are packed into first, second or pulp grades. Returns to the grower are improved when most fruits are in first grade. Many factors such as skin blemishes from sap, abrasion and temperature stress, misshapen fruit, small or large fruit size, and disease and disorders can downgrade fruit. Although many of the individual traits impacting on pack-out are minor, attention to all of the fruit quality traits during selection of parents and progeny in a breeding programme will assist in improvements in pack-out.

2.7.4 Harvest Time

A mix of cultivars with a spread of harvest times will extend the harvest season on the farm and provide a choice of cultivars to the consumer for a longer period. However, certain markets and production districts may have preferences for specific harvest times. There are mango cultivars with early through to late season production that can be used as parents in a breeding programme. Very early season production is often desired in many industries, but there are only a few cultivars that can be considered very early.

2.7.5 Disease Tolerance

There are several diseases that currently pose major restraints to production or limit the post-harvest life and transportability of the fruit. These diseases can be divided into pre-harvest and post-harvest diseases. Diseases such as malformation (complex of *Fusarium* spp.), bacterial black spot (*Xanthamonas campestris*), powdery mildew (*Odium mangiferae*), mango scab (*Elsinoe mangiferae*), mango wilt (*Ceratocystis fimbriata*), slow decline and sudden death are all restraints to economic production. Anthracnose (*Colletotrichum gloeosporioides*) and stem-end rots (*Lasiodiplodia and Dothiorella* sp.) are the primary post-harvest diseases responsible for reduced saleable life and losses of fruit. Any improvements in post-harvest disease tolerances will substantially contribute to cultivar reliability and quality.

Bacterial black spot – The susceptibility of mango cultivars to bacterial black spot varies. However, no cultivars have been identified with total resistance to this disease.

Mango wilt – Several rootstocks and scions varieties with resistance to mango wilt were identified and tested in Brazil between 1989 and 1997 (Rossetto et al. 1997). These may be a useful source of resistance as rootstocks or in crossing programmes in countries where this disease is becoming established. The cultivars 'Carabao' 'Manga D'agua', 'Espada Vemhelha' and 'Voutpa' are some of the better performing wilt resistance varieties (Rossetto and Ribeiro 1990; Carvalho et al. 2004).

Anthracnose – Anthracnose is a pre-harvest disease of foliage, twigs, flowers and fruit, and the most significant post-harvest disease of fruit. Anthracnose affects mangoes in all mango growing countries of the world. There are many reports of varying susceptibility of varieties to anthracnose but no known significant resistance to the disease. There have been many reports of mango varieties that are more tolerant to anthracnose than other (Peterson 1986; Paez Redondo 1996; Sharma and Badiyala 1999; Dinh et al. 2003) with varieties such as 'Alphonso', 'Brasamasi', 'Carabao', 'Carrie', 'Early Gold', 'Keaw', 'Kent', 'Krishan Bhog', 'Rad', 'Saigon', 'Tommy Atkins' and 'Van Dyke' being most tolerant. However, none of these varieties has true resistance or levels of tolerance that significantly reduce the use of pre-harvest and post-harvest fungicides. Inter-specific crosses between *M. laurina* and *M. indica* may improve the genetic tolerance to anthracnose. The wild mango species *M. laurina* is closely related to *M. indica* and is well adapted to wet climates and poor soil drainage. It sets fruit well and shows no signs of attack by anthracnose in areas where anthracnose prevents other mangoes from setting fruit (Bompard 1993).

Mango malformation – Mango malformation is a serious disease in many countries in the Americas, Africa and Asia that deforms shoot tips and panicles preventing the set of fruit. Currently the disease is managed, but not controlled, by cultural methods. Sharma and Majumdar (1988) used the variety 'Bhaudauran' as a source of resistance in a F1 crossing programme, but found all the progeny to be susceptible to malformation. Other varieties have been reported as having tolerance to malformation, such as 'Bhyadayam Dula', 'Samar Bahist Rampur' and 'Miam Sahib' (Ram et al. 1987), but there have been no reports of their use in breeding programmes.

Bacterial Canker – The cultivar Bombay green has been reported as having some resistance to Bacterial canker (Prakash and Srivastava 1987).

Powdery mildew – As with anthracnose and other diseases there are differences in mango cultivars responses to powdery mildew (Iyer and Degani 1997) but no known resistance.

2.7.6 Heat Treatment Tolerance

Access to many international markets require disinfestation quarantine protocols that heat the fruit for extended periods. Cultivars that can withstand heat treatments without loss of fruit quality will have a major competitive advantage in the international market.

2.7.7 Abiotic Stress Tolerance

With increasing human population in many mango producing nations, pressure on land and water resources is increasing and, as a result, mangoes are being grown on marginal soils and irrigated with poor quality saline water. Cultivars or rootstocks with tolerance to these conditions will help reduce the impact of declining conditions. The most well known rootstock with tolerance to high pH and saline water is the Israeli cultivar '13-1'(Gazit and Kadman 1980).

2.7.8 Dwarfing

Reduced vegetative vigour and reduced tree size are desirable in commercial mango production because they maximise tree productivity, reduce tree management costs and allow increased tree densities per hectare. Dwarfing can be a cultivar trait or induced in a scion cultivar by the rootstock. Traits such as high phenolic levels, higher phloem/xylem ratio and low number of new shoots giving rise to flushes have been associated with dwarfing (Singh et al. 1986; Iyer and Kurian 1992). Trunk cross sectional area have been correlated with vigour and cumulative fruit yield in mango (Reddy et al. 2003). These may be useful indicators for selection of dwarfing in a breeding programme.

Several mango cultivars have been identified as potential dwarf parents in a breeding programme, including the Indian cultivars 'Creeping', 'Kerala Dwarf', 'Janardan Pasand', 'Manjeera' and 'Amrapali' (Iyer and Subramanyam 1986; Iyer and Degani 1997). Sharma and Majumdar (1988) found that dwarfing in Indian cultivars was controlled by a recessive gene. Pinto and Byrne (1993) found that 'Amrapali' and 'Imperial' as male parents showed a high propensity to transfer the dwarf trait to their progeny. However, the canopies of the progeny were often dense or spreading. 'Creeping' has been used as a female parent in the Australian National Mango Breeding Program, effectively reducing tree vigour in it's progeny. However, small fruit size and bunch bearing are also characteristics of the progeny. The cultivar 'Vellaikulamban' used as a rootstock has been shown to reduce tree vigour and increase yields per unit canopy volume and per unit land in the cultivar 'Alphonso' (Reddy et al. 2003).

2.7.9 Fruit Flavour

Flavour in mangoes, as in other fruits, is made up from sugars, acids and volatile aroma compounds. It is the mix and concentrations of the volatile aroma compounds that give the different mango cultivars their characteristic flavours. Over 370 free forms of aroma volatile compounds have been identified in mango and 70–140 as glycosidically bound aromas. Monoterpenes and sesquiterpene hydrocarbons account for about 59% and esters about 20% of the identified compounds. Esters,

alcohols, carbonyls and lactones contribute towards the unique aroma and flavour of certain cultivars. The unique flavour of 'Kensington Pride' has been attributed to the high predominance of monoterpenes such as α-terpinolene and 'peach' flavours provided by esters and lactones (Lalel et al. 2003). Flavour preferences may be different between markets, but in many Australian markets the 'Kensington Pride' flavour is considered superior. Regardless of the flavour style, it is desirable for the flavour of a new cultivar to be robust and associated with strong aroma in ripening fruit. High flavour quality is desirable in all fruit, not just the top 10% of tree ripened fruit, if market reliability is to be achieved.

2.7.10 Fruit Shelf Life

As most of our fruit is grown a long way from the consuming markets, varieties that maintain quality after storage and transport will have an advantage by reducing retail shrinkage and maintaining consumer confidence. Many factors contribute to the shelf life and transportability of fruit such as susceptibility to post-harvest rots, resistance to blemishing and retention of flavour.

2.7.11 Fruit Size

350–400 g is the preferred size of many retailers, with smaller markets for other sizes of fruit. Size uniformity is also desirable as it contributes to higher pack-out percentages.

2.7.12 Fruit Colour

Fruit colour has three main aspects: background skin colour when ripe, skin blush colour and internal flesh colour. Reliable de-greening of the background colour during fruit ripening to a light yellow is desirable in many markets as it indicates ripeness to the consumer and lightens the overlying blush making the colour more vivid. Many current cultivars do not de-green well when grown under high nitrogen or if stored or ripened under non-optimal conditions.

The blush colour is the overlying orange, pink, red or purple colour that develops on fruit in the second half of development and it is often dependant on exposure to direct sunlight. Strong blush colour that develops with minimal exposure to direct sunlight on the majority of fruits on a tree is desirable. Blush colour is mainly the result of anthocyanin pigments that appear to be primarily the result of activation of cyaniding-3-*O*-galactaside synthesis (Berardini et al. 2005a). Tentatively identified in the peels of red-coloured mango cultivars (e.g. Tommy Atkins) are the compounds cyanidin 3-*O*-galactoside (Berardini et al. 2005a) and 7-*O*-methylcyanidin 3-*O*-β-D-galactopyranoside (Berardini et al. 2005b). Many cultivars in Asia lack

or are poorly blushed. However, the cultivars originating out of Florida are highly coloured and make good parents. Cultivars such as 'Tommy Atkins' and 'Irwin' have been used successfully to improving skin blush in progeny. Bally et al. (2006) reported broad-sense heritabilities of between 0.41 and 0.59 for colour in families with 'Tommy Atkins' and 'Irwin' as a female parent.

Internal pulp colour ranges from pale yellow-green to deep orange in a ripe fruit. The intensity of flesh colour varies with cultivar and appears to be the result of both carotenoids (Pott et al. 2003) and an orange anthocyanin (Proctor and Creasy 1969). Cultivars rich in α-carotene are characterized by a marked colour change during ripening while cultivars poor in α-carotene have less pronounced red coloration (Vásquez-Caicedo et al. 2005). The carotenoid composition varies, depending on the cultivar, geographic or climatic effects, stage of maturity, fruit processing and storage conditions during shipment (Mercadante and Rodriguez-Amaya 1998).

In addition to their aesthetic colour attributes, these pigments have antioxidant activities with many health-promoting properties, including some cancers, atherosclerosis and age-related macular degeneration (Mayne 1996; Nishino et al. 1999).

2.7.13 Fruit Internal Disorders

Internal fruit disorders are difficult to manage and are generally undetected until the consumer opens the fruit. High incidence of internal fruit disorders in a cultivar reduces the confidence of the consumer and retailers in the cultivar. The causes of internal fruit disorders are not certain but factors such as nitrogen and calcium nutrition (Burdon et al. 1991, 1992), crop load and tree vigour (Subramanyam et al. 1971; Simmons et al. 1998) and fruit maturity at harvest (Galan Sauco and Calvo 1984) have all been implicated. The incidence and severity of internal disorders varies between mango cultivars with the highest incidences reported in cultivars originating from Florida and India (Limaye et al. 1975; Oosthuyse 1993). Rane et al. (1976) reported that the cultivars 'Pairi', 'Kesar', 'Doophperda', 'Neelum' and 'Dashehari' were free from the 'spongy-tissue' form of internal breakdown. Any lowering of the incidences of internal disorders in new cultivars will contribute to consumer confidence in mangoes.

2.7.14 Fruit Shape

The shapes of the mango fruit vary from round to elongated, ovate, with shape being a characteristic trait of cultivars. Fruit shape and colour are the most recognisable features of a mango and can be important for consumer recognition of a cultivar. Longer shaped fruits and fruits with prominent beak shapes can be more difficult to handle in a mechanised sorting and packing system. Iyer and Degani (1997) reported predominant beak shape being dominant over a less pronounced beak.

2.7.15 Sap Burn

The lactiferous sap that spurts and oozes from the peduncle of mango fruit during harvest is toxic (Joel 1980) and when it comes into contact with the fruit it burns the skin causing sap burn or skin browning (Loveys et al. 1992). These blemishes reduce the visual attractiveness and value of the fruit. The sap is also toxic to human skin causing skin dermatitis on contact (Calvert et al. 1996). Avoiding and removing sap contamination is a major expense in many mango growing enterprises. Breeding mango cultivars with low sap toxicity would greatly improve both fruit quality and production efficiency. The toxicity of sap differs between cultivars, regions and weather conditions (Loveys et al. 1992; Hesse and Bowden 1995). Loveys et al. (1992) found the toxic component in 'Kensington Pride' sap to be the volatile terpinolene that has also been identified as the main contributor to the 'Kensington Prides' characteristic flavour (Lalel et al. 2003), making breeding for the reduction in sap toxicity difficult without a corresponding reduction in flavour.

2.7.16 Seedless

Seedless fruits have been developed in many fruit species, for example, table grapes, citrus and watermelon. Although seedlessness occasionally occurs naturally in mango, it is usually associated with inferior and undersized fruit (nubbins). Hormonally induced seedless mango fruits of 'Dashehari' were also undersized (Chacko and Singh 1969). Selection of seedless mango has been part of the Indian breeding programme for several years. During the 1990s, India commercially released the 'seedless' cultivar 'Sindhu' that bears medium-sized fruits with extremely thin stone and devoid of embryo and endosperm (Gunjate and Burondkar 1993). However, this variety did not have commercial success (Anonymous 2006). Recently, in Hainan Island, China, commercial scale of production of seedless mangoes using plant growth regulator treatments had made great success in domestic and export markets (Lu et al. 2006) using the cultivars 'Tainong No. 1', 'White ivory' ('Nang klang wan') and 'Guifei'('Red-golden dragon') that usually produce fruits with fully developed seeds.

2.7.17 Rootstock

The requirements for mango rootstocks are somewhat different from those for scion cultivars. Effective rootstocks should be graft-compatible with scion cultivars and also confer some beneficial effects to the scion such as dwarfing or tolerance to calcareous soils and salinity (Litz and Gomez-Lim 2002). For the ease of propagation, on a commercial scale, polyembryonic rootstock cultivars are preferred. One of the most successful rootstock selections was the cultivar '13-1' selected in Israel for its tolerance to salinity (Gazit and Kadman 1980; Lavi et al. 1993). Recent research

with tetraploid polyembryonic mango selections (Section 2.3.1) has indicated new opportunity for dwarfing rootstocks.

2.8 Breeding Techniques

2.8.1 Historical Overview

Historically, most of the current cultivars grown worldwide originated from selection of open pollinated seedlings, either as chance seedlings or specific selection and breeding programmes. A few cultivars have originated from controlled closed pollination. The systematic breeding systems used include techniques such as monoembryonic and polyembryonic seedling selection, controlled open and closed pollination.

Each of these techniques is influenced by the reproductive physiology of the mango that creates some significant challenges and limitations with some conventional breeding techniques. Factors such as the perennial flowering habit, large number of flowers per panicle, polyembryony, juvenility, polyploidy and inherent heterozygosity (Mathew and Dhandar 1997; Villegas 2002) can all potentially influence the management and success of mango breeding programmes. However, the ability to easily vegetatively reproduce clone trees allows any genetic gain to be captured in a new cultivar. The merits and drawbacks of each technique are discussed below.

2.8.2 Mutation Breeding

Natural spontaneous mutations in mango have been reported with cultivars such as ‘Davis-Haden’ originating as a sport of ‘Haden’ and ‘Alphonso’ and ‘Puthi’ originating as chimeras (Singh 1960). γ radiation and chemical induction of mutations in mango for breeding purposes has been reported to induce dwarfness, firmer flesh, higher T.S.S. and better sugar acid blends in a few of the treated plants (Sharma 1987).

2.8.3 Monoembryonic Seedling Selection

Many of the major cultivars around the world are the result of selections that were made from naturally occurring seedlings and exploited the natural out-crossing in open pollinated seedlings. Cultivars originating from Florida and India during the last centaury have often come from organised monoembryonic selection programmes. The Australian cultivar ‘R2E2’ originated from a seedling selection programme based on ‘Kent’ (Bally 1998). In monoembryonic selection programmes large numbers of seedlings from one or more desired monoembryonic maternal

parents are grown and evaluated and screened for improved genotypes. Polyembryonic maternal parents are not suitable as their seedlings are genetically similar to the maternal parent.

There are several limitations of the monoembryonic selection technique. The diversity amongst seedlings is generally low due to self-pollination in many of the seedlings. Self-pollinated seedlings cannot easily be detected until they begin to crop, so large numbers of seedlings have to be field grown and screened before suitable improved selections can be identified. In addition to the low percentage of selectable seedlings, the breeder does not have any control over the paternal (pollen) parent of the hybrid seedlings. These limitations are offset by the ease in generating large numbers of hybrids for testing. However, the high effort to reward ratio and low genetic control of this technique make it less favoured by modern mango breeders. Molecular pre-selection techniques such as MAS may be a useful way to selectively identify desirable types and avoid large field plantings with monoembryonic selection breeding.

2.8.4 Polyembryonic Seedling Selection

Ployembryonic seedling selection aims to exploit the diversity in polyembryonic populations generated by natural mutation or out-crossed zygotic seedlings. It is well known that most polyembryonic seedlings are nucellar in origin and true to the maternal parent type. However, zygotic off-type seedlings have been reported between 2% and 47% in several polyembryonic cultivars (Beal 1981; Truscott et al. 1993). The main problem with this method and the reason it has not been traditionally favoured by plant breeders is the ability to identify the zygotic embryos in the seed. Generally polyembryonic selection programmes have relied on identifying superior types within large commercial populations rather than selecting and growing zygotic seedlings for selection.

This method has been reported to produce some interesting cultivars in Florida such as ‘Alica’, ‘Herman’ and ‘Florigon’, all of which originated from seedlings of ‘Saigon’ (Knight and Schnell 1993) as well as some variations in the cultivar ‘Kensington Pride’ (Johnson 1995).

2.8.5 Controlled Open Pollination

Controlled open pollination exploits the natural out-crossing of monoembryonic cultivars with measures taken to encourage out-crossing with pollen from a desired source.

Several techniques can be used to maximise out-crossing to desired maternal (pollen) parent. Often the two parent trees are enclosed with pollinating insects in a large insect-proof cage. This technique was used by Sharma and Singh (1970) with the self-incompatible cultivar ‘Dashehari’ and other cultivars to produce the quality hybrids ‘Mallika and ‘Amprapali’. This technique has also been employed in Israel

(Degain et al. 1993). One major constraint on this technique is the requirement for synchronised flowering of the caged parent trees. An alternative method is to plant single female parent trees in close proximity and surrounded by male pollinating parents or to graft a branch of the female parent on to the male parent tree, thus, allowing a higher probability of out-crossing to the desired male parent. This technique has been successfully used in Australia by Whiley et al (1993) who produced the cultivar 'Calypso' from a cross between 'Sensation' ♀ and 'Kensington Pride' ♂.

Controlled open pollination has been one of the more popular breeding techniques used in the last 25 years, but it has several shortcomings. As with all open-pollinated techniques, high numbers of self-pollinated progeny are often produced (Lavi et al. 1998) that have to be field grown and evaluated for several years before being recognised and culled, tying up valuable land and time resources. Positive identification of the male or pollen parent is not easy without DNA fingerprinting, making statistical analysis of segregation patterns in the breeding population difficult and providing little improved understanding of mango genetics.

2.8.6 Controlled Closed Pollination (Hand Pollination)

Controlled closed pollination uses hand pollination techniques to cross two desired parents, delivering the desired pollen and excluding the un-desired pollen to the female parent flowers.

Early attempts at hand pollination involved crossing a large number of flowers on a few panicles, over several days, resulting in a very small number of hybrids due to the large natural fruit thinning of initial fruit set per panicle (Singh 1960). This technique was substantially improved by Mukherjee et al. (1961) who crossed larger number of panicles and fewer flowers per panicle. Further modification to the technique of Mukherjee et al. (1961) was suggested by Singh et al. (1980) who did not re-bag the crossed panicles after pollination. They claimed that re-bagging damaged stigmas and styles of the crossed flowers, reducing the percentage of fruit set and the risk of pollination from unwanted pollen was very low. Another modification to the Mukherjee et al. (1961) technique has been to replace re-bagging step after crossing with application of gelatinous capsules to enclose the flowers (Bally et al. 2000). This technique is currently used in the Australian mango breeding programme and is described in detail in Section 2.9.7.

The advantages of the hand pollination technique are that pollination can be carried out on any desired female parent trees with a wide range of pollen sources not necessarily located in the same site, without setting up purposely grown crossing blocks. Both parents of each hybrid generated through closed pollination techniques are identified, making statistical analysis of segregation patterns possible. Unlike open pollination techniques, resources are not wasted on growing and evaluating self-pollinated progeny.

The biggest disadvantage of this technique is the relatively low number of hybrids generated per number of panicles crossed. However, success rates are dependant on the particular parental combination used in the cross. Kulkarni et al. (2002) reported

success rates of between 0 and 122% (percentage of mature hybrid seed obtained per crossed panicle) depending on the parents involved. The highest success rates (>100%) were the bunch bearing cultivars such as 'Creeping' and 'Willard'. The reasons for the low success rates of the hand-crossing technique are not fully understood, but may include factors such as problems of incompatibility between parents and pollen viability (Dag et al. 2000; Sukhvibul et al. 2000), stigmatic receptivity and protogyny (maturation of stigmas before anthers) (Spencer and Kennard 1956) and low natural fruit set in mango.

2.8.6.1 Method of Hand Pollination

Hand pollination (crossing) is a slow process that is limited by the time of day and shortness of the flowering season. Fresh pollen is generally not available until the anthers dehisce two or three hours after sunrise and is unavailable after the filament and pollen begins to shrivel at about midday. A description of hand pollination in mango is outlined and illustrated below.

The ideal panicles for crossing are those that have minimal open flowers and between 5 and 20 light tulip shaped flower buds ready to open within 24 hours (Fig. 2.3a and b). These panicles provide an adequate numbers of flowers for crossing the next day and easy removal of all other unwanted flowering buds. More developed panicles with larger numbers of open flowers can be used, but they generally take longer to prepare and cross.

Selection and preparation of panicles is best done in the afternoon to minimise the chance of the selected flower buds opening before hand-crossing the following morning. All open flowers are removed from the selected panicles by pinching them off with fine tweezers (Fig. 2.4a). This removes all pollen sources that may

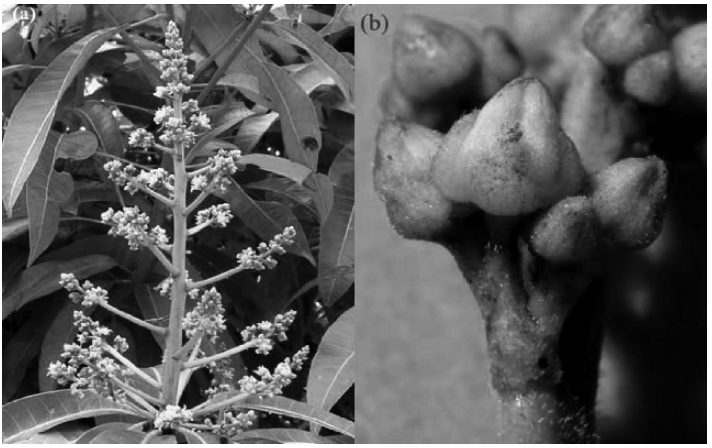

Fig. 2.3 (**a**) Mango panicle suitable for crossing with between 5 and 20 open flowers and buds ready to open within 24 hours. (**b**) Pale tulip shaped flower bud ready to open in the next 24 hours (*See* Color Insert)

Fig. 2.4 (**a**) Preparation of panicle the day before pollination by the removal of all open flowers. (**b**) Subsequent bagging to exclude pollinators with tightly woven cloth bags (*See* Color Insert)

self-pollinate any flowers that open early the next morning. The panicles are then enclosed with a tight woven cloth bag to exclude external pollen and pollinating insects (Fig. 2.4b).

Pollen is collected early on the morning of hand-crossing. It is collected from the male parent from open flowers that have not yet dehisced (Fig. 2.5a). These flowers are removed and stored in a shallow container (Petri dishes are ideal for this purpose, Fig. 2.5b). Once adequate pollen flowers have been collected they are divided into two halves with one half being sealed and kept in a relatively cool spot.

The other half is kept in a shallow dish that is placed in a well ventilated warm position to encourage pollen dehiscence, which is indicated by a change in anther colour from dark red to greyish blue (Fig. 2.6). Once the anthers have begun to dehisce, the shallow dish should be removed to a shaded position to slow subsequent dehiscence. Panicles are inspected and prepared for pollination in the early morning before the anthers on the opening flower buds dehisce. The bagged panicles are uncovered and inspected for suitable open hermaphrodite flowers that the anthers have not yet dehisced (Fig. 2.5a). Between 5 and 10 flowers are selected and retained

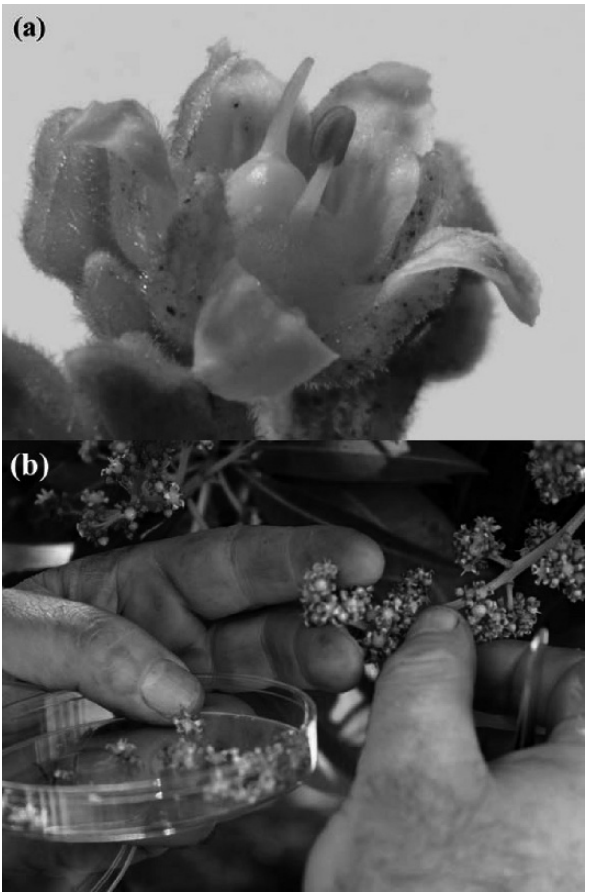

Fig. 2.5 (**a**) Freshly opened flower, prior to dehiscence of the anther. (**b**) Collection of pollen flowers for hybridisation. Pollen donor flowers should be collected at this stage of development in the early morning (*See* Color Insert)

on the panicle for crossing. All other flower buds and unwanted panicle branchlets are removed by pinching them off with fine tweezers (Fig. 2.7).

Once the panicle has been prepared by removing all unwanted flower buds, the remaining hermaphrodite flowers are emasculated to remove the stamens before the anthers have a chance to dehisce. This is done by pinching off the anthers from their supporting filaments with a fine pair of tweezer (Fig. 2.8a). Care should be taken to NOT mistake the pistil for a stamen or damage it during the emasculation operation. In between flowers, tweezers should be washed and sterilised with ethanol to minimise unwanted pollen transfer.

When the flowers on the female parent have been emasculated they are ready for pollination. This involves the transfer of pollen from the dehisced anthers of the male parent on to the stigma of the female parent (Fig. 2.8b). A pollinating flower with dehisced anthers from the male parent, collected earlier, is picked up with a

Fig. 2.6 Dehisced anther revealing grey pollen. Anther dehiscence is easily recognised by the change in colour of the anther from red to greyish blue (*See* Color Insert)

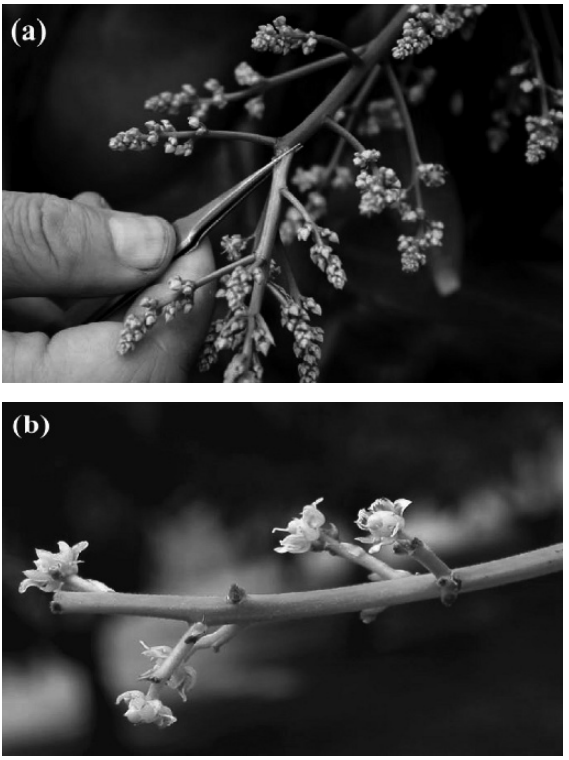

Fig. 2.7 (**a**) Removal of flower buds not selected for crossing with tweezers prior to pollination. (**b**) Panicle prepared for pollination. Only the flowers to be pollinated are retained, all other flowers are removed from the panicle (*See* Color Insert)

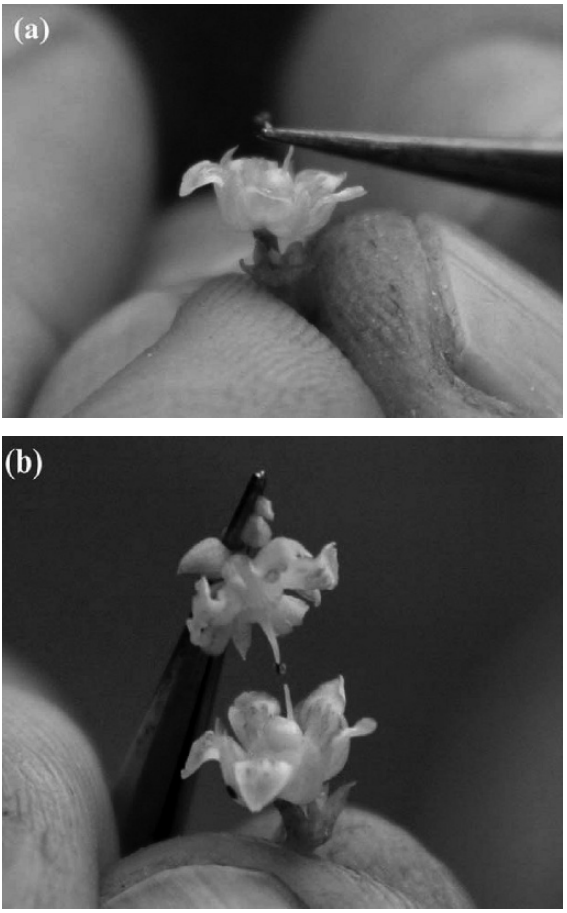

Fig. 2.8 (**a**) Emasculation of hermaphrodite flowers prior to pollination by removing the anthers from the filaments with a fine pair of tweezers. (**b**) Transfer of pollen by gently touching the dehisced anther of the male flower on to the stigma of the female flower (*See* Color Insert)

freshly sterilised pair of tweezers and the anthers are gently touched on the stigma of the female parent flower to dislodge pollen grains. One pollinating flower should not be used on more than three female flowers to ensure that an adequate quantity of pollen is transferred.

Immediately after the pollen transfer, the female flower is covered with half a gelatine capsule to prevent foreign pollen reaching the stigma and to retain humidity around the stigma (Fig. 2.9). These gelatine capsules can be removed the next day or left on to dissolve in dew water.

After pollination, a label indicating the parentage and date of the cross is tied around the base of each crossed panicle. As the hybrid nears full maturity, the fruit and panicle are covered with a cloth bag to exclude potential pests and to catch the fruit if it falls, preventing it from coming into contact with the ground and retaining it's identity.

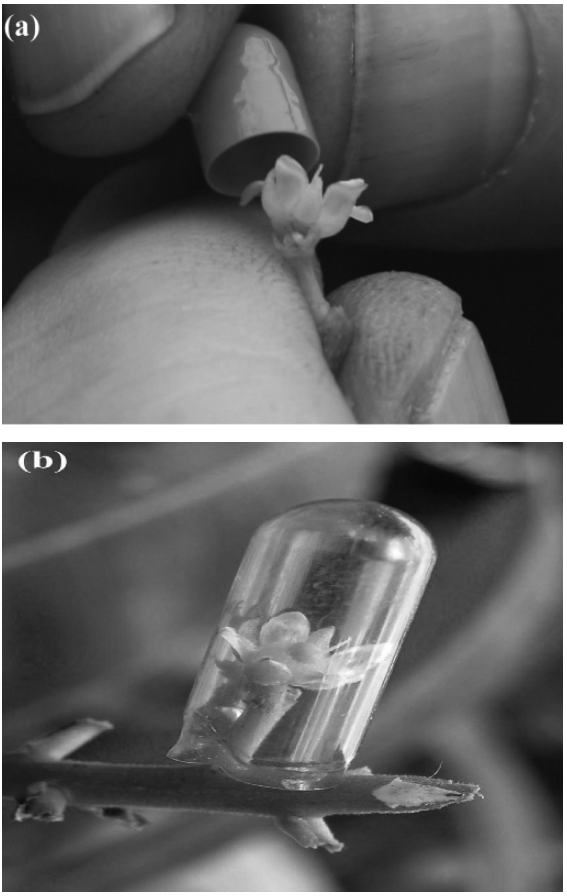

Fig. 2.9 (**a**) and (**b**) Encapsulation of female flowers after pollination to exclude foreign pollen and retain humidity around the flower for pollen germination (*See* Color Insert)

2.9 Biotechnology-Assisted Breeding

Biotechnology-assisted breeding referes to the application of molecular biology and somatic cell genetics to the improvement of plants (Litz and Lavi 1997). Use of the biotechnology-assisted breeding methods may overcome or alleviate many of the limitations discussed above, such as low fruit retention, polyembryony, hetrozygosity, polyploidy, long juvenility, large tree size and long life cycle.

Molecular biology (genetics) can assist mango breeding/improvement in several ways. In conventional breeding, marker-assisted selection can be used for the identification of parents and selection of progeny and gene cloning can be used for transforming existing mango cultivars with horticulturally important genes (Litz 2004). Other biotechnological methodologies include the efficient somatic embryogenesis and plant recovery from elite (nucellar) material, induction of random mutations in embryogenic cultures and challenging for resistance to a specific selective agent

and transformation with a gene that mediates a desired horticultural trait. On the basis of past and current research, it is probable that mutation breeding can address resistance to abiotic soil stress and some diseases, while genetic transformation can addresse the control fruit ripening, seedlessness and other diseases. (Litz, 2004). Progresses have been made on controlling fruit ripening by inhibition of expression of genes encoding ethylene biosynthetic enzymes (Cruz-Hernandez et al. 1997) and selection of several embryogenic cultures of mango that indicate some resistance to anthracnose fungal pathogen (Jayasankar et al. 1998; Jayasankar et al. 1999; Litz 2004). There are several reviews on the application of the modern biotechnology tools on mango cultivar improvement to which the readers can refer to (Litz and Lavi 1997; Litz and Gomez-Lim 2002; Lavi et al. 2004; Litz 2004).

2.10 Perspective

Despite some of the constraints and long time frames of conventional breeding in mango, many opportunities exist to produce new cultivars that have improved traits that will improve productivity, fruit quality and the economic competitiveness of mango industries. Recent and future development of molecular markers, especially those that are suitable for a high throughput MAS, should substantially improve the efficiency of the conventional mango breeding by identifying the genetic linkage between marker allele and the gene of interest. Increased use of molecular markers and other pre-selection techniques has the potential to substantially reduce selection time frames in mango breeding programmes. Identification and cloning of horticulturally interesting genes from mango and its wide relatives will enhance the rate of conversion or plant survival from germinated transgenic somatic embryos and would certainly revolutionise the genetic improvement of mango cultivar. Biotechnological developments and statistical analysis of current breeding populations are also substantially improving our understanding of the genetic traits and their inheritance in mango. These understandings make it easier for the breeder to select parents and design breeding programmes with more specific breeding goals than has been possible in the past. However, there are still significant constraints in controlled hybridisation that need to be overcome if larger breeding populations are to be generated. Studies to improve our understanding on pollination compatibility, timing and techniques can contribute to improved breeding efficiency.

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Chapter 3
Breeding Guava (*Psidium guajava* L.)

Celso V. Pommer and Katia R. N. Murakami

3.1 World Production

Guava tree (*Psidium guajava* L.) has its origin in the American tropics and is today distributed throughout the tropical and subtropical areas of the world (Kwee and Chong 1990; Gonzaga Neto and Soares 1994; Medina 1988). It belongs to the Myrtaceae family, comprising a large number of fruit yielding species (Pereira and Nachtigal 2002; Ray 2002; Kwee and Chong 1990; Subramanyam et al. 1992). The guava fruit is important for fresh consumption and for substantial industrial preservation (Maia et al. 1988). The fruit contains vitamin A and B, and are exceptionally rich in vitamin C (ascorbic acid), superior to that present in the citric juices.

According to Ellshoff et al. (1995) *P. guajava* was first named by Linnaeus in 1753. As Ruehle (1964) stated, initial references to the guava tree are from the Spanish chronicler Oviedo, from the period between 1514 and 1557, when he was in Haiti. On that occasion, Oviedo referred to the guava tree as guayabo and made considerations about the vegetative behaviour of the plants found in some areas of West Indies. Oviedo (1959) made this insightful statement about guavas: 'Fruits have many seeds that are bothersome only to those who eat the fruit for the first time. Foods with such a heavenly taste and smell just might be considered sinful'. It is believed, on the other hand, that the Spaniards transported the guava tree of the Pacific to the Philippines and India, from where it passed to the Malay Archipelago, to Hawaii and to South Africa (Soubihe Sobrinho 1951). However, there is enough scientific evidence of guava having a pre-historic anthropogenic distribution all over the Antilles (Newsom 1993).

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The species is widely cultivated for its fruit and has become naturalised in tropical and subtropical areas worldwide. The guava has been cultivated and distributed by man, birds and other animals for so long that its place of origin is uncertain, but it is believed to be an area extending from southern Mexico into or through Central America (Morton 1987). It is common throughout all warm areas of tropical America and in the West Indies (since 1526), the Bahamas, Bermuda and Southern Florida where it was reportedly introduced in 1847 and was common over more than half the State by 1886. Although the guava plant was domesticated more than 2,000 years ago, it was not until 1526 when the first commercial cultivation of guava was reported in the Caribbean islands. Later it was spread by explorers into the Philippines and India (Yadav 2006). Early Spanish and Portuguese colonisers were quick to carry it from the New World to the East Indies and Guam. It was soon adopted as a crop in Asia and in the warm parts of Africa. Egyptians have grown it for a long time and it may have travelled from Egypt to Palestine. It is occasionally seen in Algeria and on the Mediterranean coast of France.

The world production of guava is increasing. Figures presented by FAO (Table 3.1) shows an increase of more than 10% in the last 5 years.

The status in Brazil, one of the major guava producers, gives a good idea on how growers are responding to market demand. Although the total area and the production increased 45% from 1999 to 2004, in Northeast Brazil, where guava is produced under irrigation and intensive technology, it increased by 90%. In this particular case, improved varieties played a very important role, as newly released varieties, such as 'Paluma', were used to establish new orchards. In India, the largest world producer, guava is produced in more than 150,900 ha (Fruits 2006) yielding over 1.6 million tons, but productivity is as low as 10–12 t/ha, due to poor management and post-harvest losses (Khushk and Lashari 2006).

Table 3.1 World production of Guava by country (1,000 t)

Country	2000	2001	2002	2003	2004
Total	3646.2	3792.2	3952.9	3984.8	4035.5
India	1710.5	1631.5	1715.5	1700.0	1700.0
Pakistan	494.5	525.5	550.0	580.0	600.0
Mexico	254.2	263.4	283.3	299.2	317.0
Brazil	117.6	281.1	300.0	300.0	300.0
Egypt	216.8	228.8	243.9	231.2	230.0
Thailand	170.1	154.4	160.0	160.0	160.0
Colombia	130.6	149.6	145.0	145.7	154.7
Indonesia	137.6	138.1	138.1	138.1	138.1
Venezuela	120.0	120.0	120.0	120.0	120.0
Sudan	96.3	100.0	100.0	100.0	100.0
Bangladesh	48.0	49.0	49.9	50.9	51.8
Vietnam	38.5	37.8	34.0	35.0	35.0
Malaysia	11.7	13.0	13.1	24.8	28.9
Others	100.0	100.0	100.0	100.0	100.0

Source: FAO

Mexico is one of the major guava producers in the world with an increasing crop that surpassed 23,000 ha in 2003 (SIAP 2003) (Table 3.2). Others are Pakistan, Taiwan (6,644 ha as of 1999 and yielding 20 to 35 t/ha), Thailand, Colombia and Indonesia. Other tropical countries plant guava on parcels that vary along the years, such as Cuba and Venezuela (Table 3.2). Guava is also planted in smaller scale in other countries: Malaysia 1,641 ha in 2001 (16,861 t), Australia and South Africa (Table 3.2).

According to the South African Guava Producers Association, there are two main production areas: Limpopo/Mpumalanga (in the North of South Africa) producing around 10,000–15,000 tons of guava (puree or juice) and Western Cape (in southern South Africa) producing around 25,000 tons of guava mainly as fresh fruit. Area under guava in this region was 440 ha in 2002 and 500 ha in 2005. In Vietnam, guava is planted mainly in the Mekong delta region on 2,000 ha. Often, many farmers inter-plant guava with other fruit trees like king orange and pummelo.

In the USA, Florida and Hawaii have a very limited area devoted to guava. Even so, acreage has declined on the islands, down from a total of 125 farms that grew guava on 376 ha (including new orchards) in 1992, amounting to $1,896,000.

United States is a major importer of guava products (paste, puree, jams) and there was an increase in the import from 2002 to 2004. United States imports mainly from Brazil, Dominican Republic, Ecuador and Mexico (Table 3.3). Although international trade in tropical fruits continues to be dominated by pineapples, significant growth in both the volume and value of exchange of other tropical fruits has developed in recent years, particularly mango and, to a lesser extent, avocados, carambola, guava, lychee, mangosteen, passion fruit and rambuttan (FAO 2002). Most of the recent growth in the tropical fruit trade is based on expanded crop areas specifically intended for export.

Table 3.2 Guava: World area (A, hectares) and production (P, tones)

Country		2000	2001	2002	2003	2004	2005
Mexico	*A*	20,619	20,441	22,763	16,089	16,184	n,a,
Colombia	*A*	n.a.	n.a.	n.a.	n.a.	n.a.	16,124
Cuba	*A*	4,609	5,253	6,019	7,267	7,991	7,312
	P	17,092	23,206	28,454	40,052	52,670	47,878
South Africa	*A*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	P	21,911	25,179	26,637	22,879	17,645	n.a.
USA (Hawaii)	*A*	275	247	222	214	202	260
	P	7,212	6,940	4,400	3,039	3,674	3,674
Brazil	*A*	14,354	14,387	16,066	17,776	18,826	16,399
	P	n.a.	281,102	321,127	328,747	408,283	345.533
Pakistan	*A*	60,200	63,400	64,300	62,700	61,600	63,471
	P	494,500	525,500	538,500	531,600	549,500	571,800
India	*A*	150,000	150,000	190,000	n.a.	n.a.	151,000
	P	1,710,000	1,630,000	1,680,000	1,700,000	1,700,000	1,710,000

Sources: IBGE (Brazil); SIEAP/SAGARPA (Mexico) (SIAP, 2003); Statistics, Ministry of Agriculture, Cuba; South Africa Department of Agriculture (adapted); USDA – National Agricultural Statistics Service; Plan Frutícola Nacional (Colombia)

Table 3.3 U.S. guava imports: quantity, by country, 2004

Country	Quantity			
	Guava – paste, puree	Guava – prep. or preserved	Guava – jams	Guava, Mango and Mangosteen – dried
	Metric tons			
Brazil	1,836	353	264	0
Colombia	419	77	2	84
Costa Rica	0	97	379	38
Dominican Republic	624	1,367	63	0
Ecuador	248	1,591	3	0
Fiji	17	0	0	0
France	3	8	0	1
India	533	267	0	11
Malaysia	0	259	0	2
Mexico	495	660	0	700
Netherlands	0	0	0	0
Philippines	141	0	2	2,872
Singapore	0	0	0	6
South Africa	33	262	0	19
Thailand	1	355	0	1,387
Venezuela	0	0	0	0
Others	3	100	1	146
Total	4,352	5,397	713	5,266

Source: U.S. Department of Commerce

3.2 Botanical Aspects

In guava, flowers are white, hermaphrodite, solitary or in 2–3 flowered cymes, emerging in the axils of the leaves (Fig. 3.1). According to Soubihe Sobrinho (1951), however, only the flowers located between the middle and the base of the branch have larger probability of producing fruits. More than three flowers can appear besides the usual two or three floral buttons, but it was observed that not always all produce fruits. The calyx is entire in the bud, splitting into 4–6 sepal lobes 1–1.5 cm long, reflexed, pubescent and persistent. The corolla consists of 4–5 petals that are white, obovate, concave reflexed and 1–2 cm long. The stamens are numerous and inserted in rows on a disc, 1–2 cm long with white filaments and yellow anthers that dehisce longitudinally (Kwee and Chong 1990). The stigma is capitate, greenish yellow, 1.5–2 cm long with a filiform style arising from a 4–5 locular ovary. The guava flower has a superior calyx with 5 lobes and the corolla of 6–10 petals arranged in 1 and 2 whorls (Subramanyam and Iyer 1993). The androecium consists of 160–400 thin filaments carrying bilobed anthers closely packed together. The gynoecium consists of an inferior ovary, syncarpous with axile placentation and subulate terminal style. The style is smooth and red at the summit. It is larger than filaments, but bent over stamens in bud stage. The stigma is exserted above the stamens, thus self-pollination without the help of external agency is rather uncommon.

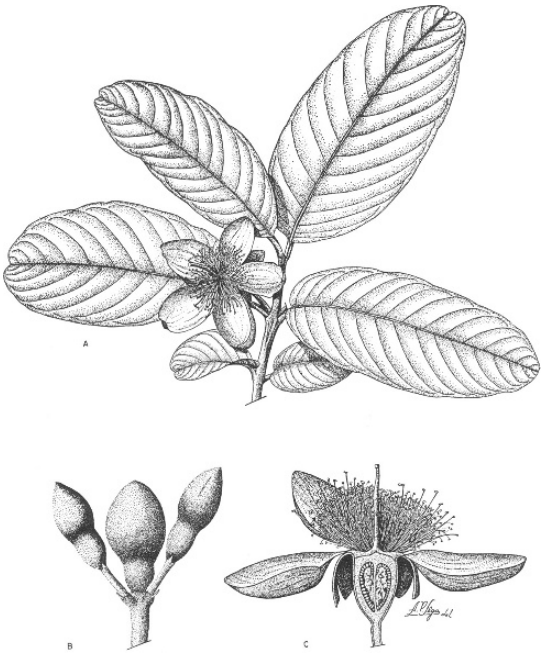

Fig. 3.1 **A** – frontal view of a guava flower in current year shoot; the five petals and the numerous stamens can be seen. **B** – guava inflorescence (cyme) with three buttons and a dichasium structure. **C** – flower in longitudinal section view with numerous ovules inside the ovary, persistent sepals, stigma with a slightly conic shape, large number of stamen and the petals typically boat-shaped. (from Soubihe Sobrinho 1951)

About 30 days are required from flower bud differentiation to complete the development up to calyx cracking stage (Subramanyam and Iyer 1993). The flower bud, when fully developed, has two distinct parts, namely, proximal ovoid adnate and distar free part that is ovoid or round and slightly pointed at apex. The cracking of the calyx occurs nearly 24 hours before flower opening. Sehgal and Singh(1967), however, observed that the calyx splits anywhere between 13 and 26 hours before flower opening.

It is observed that the fruit originating from the central floral button almost always presents faster development. This characteristic is of great importance because it can signal the correct way to practice the thinning of fruits. This is valuable in the evaluation of germplasm accessions. Observation and knowledge of such traits can guide a programme of genetic improvement, especially those pursuing derivation of varieties through recombination breeding. Guava prefers cross pollination that can vary from 25.7 to 41.3%, with an average of 35.6% (Soubihe Sobrinho and Gurgel 1962). Singh and Sehgal (1968) made a contradictory observation that guava is self-pollinated. Medina (1988) observed 62–82% open pollination. Domestic bee, *Apis mellifera*, is the main pollinating agent. While studying the natural fruit set, variations were registered from 22 to 75%, verified in cultivar Lucknow-49 (Soubihe Sobrinho 1951; Medina 1988).

According to Subramanyam et al. (1992), it takes 45–51 days to complete development of flower bud from 2 mm to full bloom during winter. Flower development in *P. quadrangularis*, *P. molle, P. cattleianum* and *P. friedrichsthalianum* take 36–45 days. Flowering in all the cultivars of *P. guajava* occurs three times a year as in *P. quadrangularis*. In *P. friedrichsthalianum* few flowers are observed between two flushes. Fruit set varies between 40 and 85.7% in *P. cattleianum*. Among the varieties of *P. guajava*, it ranges between 30.4 (cv. Pear Shaped) and 80% (cv. Allahabad Safeda and Apple Colour).

One of the most critical botanical characteristics of guavas is that the flowers are always borne on newly emerging vegetative terminals irrespective of the time of the year (Shigeura and Bullock 1976). Consequently, blossom bud formation and subsequent fruit set can be very erratic during the year or between years, depending on the rainfall pattern and the availability of fertiliser and water. However, trees can possibly be exploited by cultural manipulation to flower and fruit when desired. Under temperate conditions, there is only one fruit season a year with little choice. Irrespective of the time of the year in the tropics, new vegetative growth on guavas can be induced in several ways. The easiest and most common is by pruning the branches in a manner so that the apical dominancy of the pruned branch will not be disrupted. This is done by taking out other branches by cutting at the junction to the main branch. The duration of flowering in each of the two peak flowering seasons is 35 to 45 days in India (Ray 2002). The fruit bud differentiation is practically continuous throughout the year except during winter.

3.3 Genetic Resources

The guava tree belongs to the Myrtaceae family, comprising more than 70 genera and 2,800 species. The Myrtaceae family also includes other agriculturally important plants that yield economic products such as aromatic spices (clove, cinnamon, allspice), aromatic oils (eucalyptus), ornamental plants (myrtle, Callistemon) and a number of fruits (roseapple, Surinam cherry, Java plum, wax jambu, feijoa and many others) (Kwee and Chong 1990). The genus *Psidium* presents about 150 species, among which stand out *P. Guajava* L. (guava, $2n = 22$), *P. cattleyanum* Sabine (sweet, beach or crown araçá) and *P. guineense* Swartz or *P. araça* Raddali (true or acid araçá) (Pereira 1995).

Description of *Psidium* species is scattered throughout a number of papers and authors, but from the breeders perspective, the essential knowledge is presented by Soubihe Sobrinho (1951), Kwee and Chong (1990), Subramanyam and Iyer (1993), Gonzaga Neto (1999), Ray (2002) and Pereira and Nachtigal (2002). Also Ellshoff et al. (1995) presented an annotated bibliography on *Psidium*, while treating guava as a forest weed in Hawaii.

Psidium was described as a member of the pimentoid sub-tribe of the Myrtaceae having a C-shaped or uncinate embryo, hard or bony seeds and the calyx splitting between the lobes at anthesis (McVaugh 1968). Except for a few species that have become widespread through cultivation, most species of the genus occur as native

plants. The greatest diversity of species is from central and southeastern Brazil, but there are also a good number (about 15) from northern South America, quite a few from the West Indies, a handful from continental North America and a scattering of peripheral species from the Andes and southern Brazil. Numerous species described from central and southeastern Brazil has not been adequately studied since his time. Contrary to what other authors have accepted as a distinguishing characteristic, McVaugh found no sharp distinction in *Psidium* between groups of species with closed buds and those with open buds.

As a basis for hybridisation and improvement of cultivated *P. guajava*, Seth (1963) established comparative studies of the floral morphology, floral biology, incompatibility, cytology, embryology and seed development of *P. guajava*, *P. guineense*, *P. chinense*, *P. molle* and *P. cattleianum* var. lucidum. Floral biology of all the species was reported to be very similar to one another. *P. cattleianum* differed slightly in several ways from the other species. However, the author stated that maturation of floral buds and fruits was most rapid in this species; anthers dehisced after anthesis rather than before as in the other species; pollen had the lowest viability; optimum temperature for pollen germination was lower (25 °C as opposed to 30 °C for the other species); pollen longevity was shortest, the stigma becoming receptive the day the flower opens, remaining so for up to 72 hrs. (in other species, the stigma became receptive the day after anthesis and remained so up to 32 hrs.); when *P. cattleianum* var. lucidum and *P. guajava* were crossed, the fruits were seedless; and *P. cattleianum* var. lucidum was reported to be octaploid (as compared with *P. molle*, which was tetraploid and the other above-listed species, which were diploids of $2n = 22$).

Seedless varieties were reportedly common in *P. guajava* as stated by Seth (1959). The seedless trait is related to many factors, of which self-incompatibility and chromosomal abnormalities were considered to be the major ones. The variety 'Seedless' was diploid with $n = 22$. Both embryo sac and pollen grains were found to be functional, but the percentage of viable pollen grains was low. The meiotic division was highly abnormal showing monovalent and bivalent laggards as well as bridging of chromosomes, indicating the hybrid origin of the variety and explaining the low pollen grain fertility.

The chromosome number of *P. guajava* was reported as $2n = 22$ (Soubihe Sobrinho 1951; Kumar and Ranade 1952), but the level of ploidy was verified. Kumar and Ranade (1952) mentioned a seedless variety found to have a somatic complement of 33 chromosomes, which appeared to be the first record of triploidy.

In this matter, Hirano (1967) reported the following results obtained in his study for chromosome counts: *P. cattleianum* ($2n = 77$; based on two specimens); *P. cattleianum* var. lucidum ($2n = 66$; based on one specimen); *P. cujavillus* ($2n = 44$); *P. guineense* ($2n = 44$); *P. friedrichsthalianum* ($2n = 44, 66$); *P. polycarpum* ($2n = 22$); *P. guajava* several cultivars ($2n = 21, 22, 24, 25, 33$). Hirano (1967) also observed that pollen of *P. cattleianum* var. lucidum could not be germinated; *P. cattleianum* and *P. cattleianum* var. lucidum could be crossed in both directions; *P. guajava* could not be crossed with *P. cattleianum* or with *P. cattleianum* var. lucidum. *P. cattleianum* and *P. cattleianum* var. lucidum could not be crossed with *P. guineense*, *P. cujavillus* or *P. friedrichsthalianum*.

Polyploidy within *P. guajava* was reportedly uncommon but the genus *Psidium* itself is represented by di-, tetra-, hexa- and octoploid species (Hirano and Nakasone 1969[a]). For *P. guajava* and *P. polycarpum* $2n = 22$; for *P. guineense* and *P. cujavillus* $2n = 44$; for *P. friedrichsthalianum* $2n = 66$. Although reports of $2n = 88$ have been made for the chromosome number of *P. cattleianum* and its botanical form *P. cattleianum* f. lucidum, two plants of *P. cattleianum* were found to be heptaploid with $2n = 77$ and three plants of *P. cattleianum* f. lucidum were hexaploid with $2n = 66$ in this study. *P. guineense* and *P. cujavillus* were introduced into Hawaii as such, but similarities in chromosomes as well as in vegetative characters between these species casts some doubt as to their identities. *P. cattleianum*, *P. cattleianum* f. lucidum, *P. guajava*, *P. guineense*, *P. cujavillus* and *P. friedrichsthalianum* were subjects of pollen germination and crossing studies by Hirano and Nakasone (1969b). Pollen of *P. guajava* (cultivars used had $n = 22$ and $n = 33$) generally had high germination rates, which were higher than those for species with higher chromosome numbers. *P. cattleianum* and *P. cattleianum* f. lucidum, with reports of $n = 88$, were sometimes found to be heptaploid (*P. cattleianum*, $2n = 77$) and hexaploid (*P. cattleianum* f. lucidum, $2n = 66$). Pollen of *P. cattleianum* showed 32% germination, while that of *P. cattleianum* f. lucidum failed to germinate. Pollen tubes of *P. cattleianum* were shorter than those of other species.

3.3.1 Cultivars

Although selective breeding of guava cultivars started almost a century ago, the easiness of plant propagation through seeds hindered preserving improved cultivars without significant changes of their attributes. Only after establishment of good cloning methods such as rooting herbaceous cuttings of guava, cultivars started to be well preserved and maintaining the original characteristics.

Many breeding programmes in the world have released improved guava cultivars (Table 3.4) but the most common way of getting new varieties is through growers' actions, such as identification of outstanding plants in their orchards and their propagation. This is possible because of the great diversity in open pollinated plants used to form orchards. There are probably more than 400 guava cultivars around the world, but only a few dozen are responsible for the majority of plantings. As stated by Subramanyam and Iyer (1993) and Pathak and Ojah (1993), the description and nomenclature of guava varieties is often confusing. Usually new selections are named according to shape of the fruit, skin colour, flesh colour and after the place of origin.

Allahabad Safeda: Fruits are big in size, round, smooth skin, white flesh, soft, firm, light yellow and, on ripening, develops very sweet taste, pleasant flavour and a few seeds. It is the most popular variety in India and other countries.

Beaumont: Selected from a seedling population derived from fruits found in Halemanu, Oahu, Hawaii. Medium to large, roundish fruits weighing up to 240 g.

Table 3.4 Commercial cultivars of guava in the world

Country	Cultivars
Australia	Allahabad Safeda; Beaumont; Lucknow-49; Ka Hua Kula
Bangladesh	Swarupkathi; Mukundapuri; Kanchannagar; Kazi
Brazil	Paluma; Rica; Pedro Sato; Kumagai; Sassaoka; Ogawa; Yamamoto; XXI Century
Colombia	Puerto Rico; Rojo Africano; Extranjero; Trujillo
Costa Rica	Tai-kuo-bar
Cuba	Enana Roja Cubana; EEA 1–23
Egypt	Bassateen El Sabahia; Bassateen Edfina; Allahabad Safeda
India	*White fleshed*: Allahabad Safeda; Apple Colour; Lucknow-42; Lucknow-49; Safeda; Karela; Seedless; *Red Fleshed*: Lalit; Hybrid Red Supreme; Red-fleshed; Benarasi; Sardar; Chittidar; Harijha; Arka Mridula; Arka Amulya
Malaysia	Kampuchea (Vietnam, GU8); Hong Kong Pink; Jambu Kapri Putih; Maha 65; Bentong Seedless (Malaysian S.); Taiwan Pear
Mexico	Media China; Regional de Calvillo; China; la Labor; Acaponeta; Coyame
Puerto Rico	Corozal Mixta; Corriente; Seedling 57-6-79
South Africa	Fan Retief; Frank Malherbe
Taiwan	Tai-kuo-bar
Thailand	Glom Sali; Glom Toon Klau; Khao Boon Soom
Vietnam	Xa ly nghe; Ruot hong da lang; Xa ly don
USA (Hawaii)	Beaumont; Pink Acid; Ka Hua Kula

Pink flesh, mildly acid and seedy. Excellent for processing. Somewhat susceptible to fruit rots. Tree vigorous, wide spreading and very productive.

Lalit: Recently released, fruits are medium sized (185 g) with attractive saffron-yellow colour and red blush. Its flesh is firm and pink with good blend of sugar and acid. It gives 24% higher yield than the popular variety 'Allahabad Safeda'.

Xa ly nghe: Pear-shape, 260 g, rough skin, flesh thickness 1.5–1.6 cm, sour-sweet taste, few to moderate seediness and white.

Ruot hong da lang: Pear-shape, 400 g, smooth skin, flesh thickness 1.3–1.4 cm, acridish sweet taste, few to moderate seediness and pink.

Xa ly don: Spheroid, 270 g, roughish skin, flesh thickness 1.4–1.5 cm, sourish sweet taste, few to moderate seediness and white.

Paluma: Seedling from open-pollinated Rubi-Supreme (UNESP, Brazil). Highly productive plants (more than 50 t.ha-1), vigorous, good tolerance to rust (*Puccinia psidii* Wint.). Fruits are large (over 200 g, even in not-thinned plants), pyriform, smooth surface, yellow colour in ripe fruits, pulp of an intense dark red, firm, thick (1.3–2.0 cm), nice flavour due to high sugar content ($\pm 10°$Brix) and few seeds. Most-planted cultivar in Brazil.

Rica: Seedling from open-pollinated Supreme (UNESP, Brazil), vigorous and highly productive plants (more than 50 $t.ha^{-1}$), oval to pear-shaped with an average weight (100–250 g), green-yellowish peel, slightly rough, red pulp, thick and firm, very pleasant flavour (11 °Brix) and low acidity. Few and small seeds.

Pedro Sato: Cultivar selected by growers from open-pollinated orchards, probably from 'Red Ogawa N° 1', in Rio de Janeiro, Brazil. Vigorous plants with

relatively good yields, fruits slightly oval, good appearance (150 to 280 g), sometimes reaching 400 g in thinned branches, very rough peel, pink pulp, thick and firm, pleasant flavour and few seeds. At present, it is the table cultivar with rough peel most planted in São Paulo (Brazil).

Sassaoka: Originated from a seedling of Common Red, in Va1inhos (Brazil), large fruits (weight superior to 300 g when in thinned plants), rounded, light-pink, thick and firm pulp and few seeds.

XXI Century: Recently released (2003), it was obtained from a controlled cross between Supreme-2 and Paluma (UNESP, Brazil) presenting a very productive plant with a short cycle (130 days from bloom to harvest), large fruits (average 200 g) with thick pulp (160 mm), rosy-red, great flavour and with little and small seeds (1.3 g/100 seeds).

Tai-kuo-bar: Introduced from Taiwan, it is a table guava with large and roundish fruits, weighing 400 to 800 g in average, white pulp, juicy and crunchy and for fresh consumption.

India, the world's largest guava producer, relies on well established and very effective breeding programmes. For instance (Guava Technical 2006), at Fruit Research Station, Sangareddy, Andhra Pradesh, 2 hybrids, Safed Jam and Kohir Safeda were selected from reciprocal crosses involving Allahabad Safeda and Khoir, were released. These hybrids have been recommended for semi-arid tropical areas and have also been found suitable for juice. Subramanyam and Iyer (1993) reported that at Horticultural Research Station, Saharanpur, efforts to obtain varieties (Singh 1953) having good fruit quality and yield resulted in a superior selection, Sol, having good fruit shape, few seeds, sweet taste and high yield (Singh 1959). At Central Institute of Horticulture for Northern Plains, Lucknow, a large germplasm was introduced and evaluated for morphological characteristics, fruit quality and yield. Evaluation of 20 varieties indicated that Lucknow-49 was the best (Chadha et al. 1981).

In Brazil, pioneer work was carried out by Soubihe Sobrinho at the IAC (Agronomic Institute of Campinas) who established the basis for all subsequent breeding work involving flower biology, rate of natural crossing and other fundamentals (Soubihe Sobrinho 1951; Soubihe Sobrinho and Gurgel 1962). The first Brazilian variety may have been IAC-4 most likely resulting from a cross between a seedless and a seeded variety with round and small fruits (100–160 g).

3.4 Breeding Objectives

Nakasone and Paull (1998) indicated that the fact of being a fruit with a lot of seeds makes guava suitable for controlled hybridisation. The same authors affirm that resulting progenies of open pollination can be appropriate for development programmes of cultivars. The selection criteria are:

I – Fruits: (a) large size (200–340 g) with few seeds and thick pulp; (b) white pulp for table and dark rose for industry; (c) flavour and aroma characteristics of the fruit;

(d) content of total soluble solids superior to 10%; (e) acidity from 1.25 to 1.50% in those destined for processing and from 0.2 to 0.6 for table; (f) content of vitamin C equal or larger to 300 $g.kg^{-1}$; (g) minimum number of stone cells (probably the ones that make a stir of hardness in the pulp); (h) good post-harvest quality; and (i) resistance to diseases and insects that damage the fruits.

II – Plants: (a) vigorous trees, with a crown widely opened and low development in height; (b) resistance to pests and diseases; (c) high production; and (d) dwarfing rootstocks.

Gonzaga Neto (1999) reported that the guava breeding programme, at Empresa Brasileira de Pesquisa Agropecuária Semi-Árido (www.cpatsa.embrapa.br) in Brazil, includes the following objectives: (a) collect, introduce, characterise and select guava genotypes with defined and appropriate characteristics to production; (b) select genotypes with higher productive potential and with resistance mechanisms to pests and diseases; (c) establish important botanical descriptors for the guava tree, seeking to eliminate redundancies in data collection; (d) to maintain collection of guava tree genotypes in strategic areas of development; and (e) to select and diffuse guava tree genotypes, seeking the formation of commercial orchards and supply of elite material for other improvement programmes and nurseries.

Kwee and Chong (1990) reported that, in general, the attributes of a good commercial cultivar are:

Good size – greater than 7 cm in diameter
Consistent high yield – about 40–60 t/ha/year
Pleasant flavour and aroma
Sweet to mildly acid
Smooth – textured and palatable, with little stone cells
Thick flesh with a small seed core or seedless
Deep pink flesh (rich in vitamin A)
Soluble solids around 9–12%
Resistant to pests and diseases.

Pereira and Nachtigal (2002) presented an extensive list of guava breeding objectives carried on at UNESP/Jaboticabal (Brazil)

Fruit, external aspect:

1.1.1 Medium weight superior to 100 grams in no-thinned plants

Oval shape, with short neck
Halos of medium and/or small size
Green-yellowish or yellow peel when ripe
Resistant to transport and good keeping quality
Fruit, internal aspect:

1.1.2 Pulp colour rosy or red

Ratio pulp/total weight superior to 70% and pericarp thickness superior to 100 mm
Absence or few stains in the pericarp and absence of stone cells
Few seeds and seeds of small size

Table 3.5 Comparison between the characteristics of the fruits of two guava varieties and the desirable characteristics listed in the literature

Characteristic	1	2	'Rica' (3)	'Paluma' (3)
Fruit diameter (cm)	7.62	8.4–8.9	6.5–8.0	8.0–10.0
Cavity diameter (cm)	3.81			
Fruit weight (g)	196–280	224–672	100–160	140–250
Seeds (%)	1–2			4.96
Pulp colour	Dark pink	Dark pink	Red	Intense red
Soluble solids (%)	9–12	9–12	10.9	
pH		3.3–3.5	3.72	
Vitamin C (mg/100g fruit)	≥ 300	≥ 300		
Stone cells	Few			
Purée ratio (%)		90		93.76

(1) Hamilton and Seagrave-Smith (1954); (2) Boyle et al. (1957); (3) Pereira (1984)

Fruit organoleptic traits and contents:

Total soluble solids (SST) superior to 10° Brix and with ratio SST/TA superior to 11.7
Vitamin C content around 100 mg of ascorbic acid per 100 g of pulp
Pleasant flavour and aroma, remaining in the industrialized products.
Plants:

Productive with a minimum yield of 30 $t.ha^{-1}$
Resistant or tolerant to rust (*Puccinia psidii* Wint.)
Low and open crown

Table 3.5 shows desirable characteristics in guava fruits, according to Hamilton and Seagrave-Smith (1954) and Boyle, Seagrave-Smith, Sakata and Sherman (1957), side by side with the characteristics of the fruits of two varieties, Rica and Paluma, released some 20 years ago (Pereira 1984). Pereira (1984) selected, in segregating populations from seeds of open pollination, the genotypes that gave origin to cultivars Rica and Paluma, respectively derived of the varieties Supreme and Rubi Supreme.

Reddy et al. (2006) suggested the future line of work for widening the genetic base for effective breeding through inter-varietal hybridisation involving less seeded triploid varieties with those of high yielding, better keeping quality and less seed content. He stated that emphasis should also be given to breed scion and rootstock separately for abiotic/biotic stress situations.

3.5 Breeding Techniques

Most of the improvement programmes are based on controlled artificial pollination, using crossings among plants that present characteristics of interest for obtaining new cultivars. Plants crossed may be of the same species, in the inter-varietal crosses or from different species, constituting the inter-specific crosses. The accomplishment of controlled crossings depends largely on the adequacy of technique employed for pollen collection. São José and Pereira (1987) observed that flower

emasculation, removal of anthers, sepals and petals, when the calyx ruptures, prevents selfing. The pollen grains are viable from the phase of developed floral button to the phase of totally open floral button (blossom) and it is advisable to accomplish pollination immediately after the emasculation.

According to Pereira and Nachtigal (2002), the first step of the work is the selection of the parents, in order to make possible the combination of favourable characters in the descendants to be selected. Once the crossings are programmed, during the blossom period, collection and conservation of pollen from male parents is to be provided. During the first hours of the morning, recently opened flowers are collected from the male parent, displayed into cardboard boxes and dried in the shade for 1–2 hours. Stamens are separated and dried for 3–4 hours at 35°C. Material is passed trough 16-mesh sieve and the collected pollen is kept on glass vessels at temperatures not higher than 25°C. Soon afterwards, during the rupture of the sepals, the emasculation of female parents is done followed by pollination. Pollination must be repeated on the two subsequent days to assure success. Usually, to obtain 200 seeds, it is necessary to pollinate about 20 flowers that can have 2–5 fruits.

Soon after pollination, fruits are labelled and protected with water repellant paper bags. Harvest should be accomplished when fruits reach the stage of maturity because the seeds turn physiologically ripe before completely ripe. The seeds should be dried in shade, treated with fungicide and conserved in paper bags. Sowing can be made in 3 liter plastic bags, with two or three seeds per unit. If all seeds happen to germinate, only one plant must be allowed in each bag; the others should be carefully transplanted to other containers. During plant development, special care should be taken in management and identification.

When the flower buttons reach their maximum development, the sepals begin breaking up in several points, signalling the beginning of anthesis. The following day, at around 6 a.m., for approximately an hour, the gradual opening of almost all the buttons begins. (Soubihe Sobrinho 1951). The hour at the beginning is variable and depends on diurnal temperature. Ray (2002) stated that anthesis starts at 4.00 a.m. and continues till 10.00 a.m., the peak opening occurring between 5.00 and 7.00 a.m.

According to Soubihe Sobrinho (1951), the first insect to visit the guava tree is the bee (*Apis melifera* L.). During anthesis, bees fly over the tree, butting against the petals in order to remove that obstacle in search of pollen. It is said in ‘search of pollen’ because exams done on some flower buttons did not reveal the existence of nectar glands. It can be said that bees assist in opening the flower, though anthesis happens in a lesser time than necessary for it to usually take place.

The flowers are immediately visited and pollinated by that insect. As diurnal temperature increases, other insects may appear.

Dehiscence of anthers and receptivity of the stigma occur just after the opening of the flower. As stated by Souza (1998), pollen viability was 99.59% and frequency of diads and triads was 0.25%.

The flower buds that open the next morning show a cracking of the calyx on the previous day, nearly 24 hours in advance. São José and Pereira (1987) verified

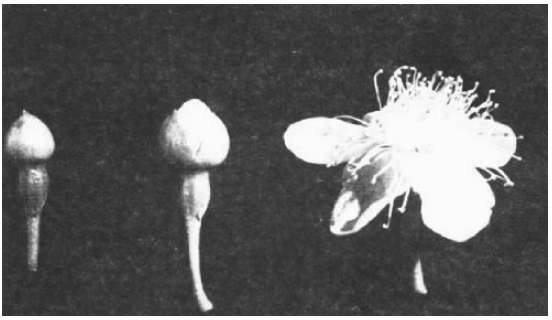

Fig. 3.2 Guava flower: developed button; button showing callyx rupture; open flower (São José and Pereira 1987)

the most adequate stage of flower development for pollen grain collect and the most efficient and safe pollination technique in controlled crosses of guava aiming to obtain specific knowledge for genetic breeding research work. They tested the efficiency of pollen obtained from closed flowers with calyx rupture and previously packed open flowers (Figs. 3.2 and 3.3), associated with pollination just after emasculation or 24 hours after emasculation. The authors concluded that emasculation of

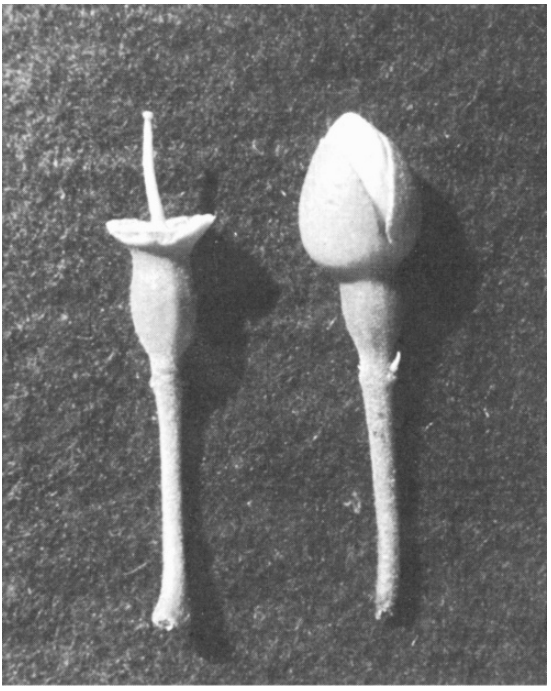

Fig. 3.3 Guava flower: emasculated button; button showing callyx rupture (São José and Pereira 1987)

guava flowers with calyx rupture and total elimination of anthers, sepals and petals prevents any possibility of self-pollination; pollen grains showed to be viable at the three tested stages of flower development; pollination is more effective when it is done 24 hours after emasculation when results are evaluated through fruit set percentage and average seed number.

Though anthesis starts at 4.00 a.m. and continues till 10.00 a.m., the peak opening occurs between 5.00 and 7.00 a.m. The dehiscence of anthers starts 15–20 minutes after the opening. In majority of the cultivars, peak dehiscence time is 6.00–8.00 a.m. In *P. Friedrichsthalianum*, peak dehiscence occurs between 7.00 and 9.00 a.m., while in *P. cujavillus* and *P. cattleianum* it is between 9.00 and 11.00 a.m. Viability of the freshly collected pollens varies from 42 to 95% depending upon the varieties. Seedless cultivars, in general, have less than 50% pollen viability whereas the seeded varieties like Chittidar or Allahabad Safeda show over 90% pollen viability at the time of dehiscence (Ray 2002).

Pollen grains of *P. guajava* L., *P. guineense* Swartz, *P. molle* Bertol, *P. chinese* Lodd and *P. cattleianum* Sabine var. lucidum remain viable for 1 day under field conditions but are viable for 90–135 days at low temperature (0–4.5 °C) and low relative humidity (0–25%). Pollen grains of commercial cultivars like 'Chittidar' could be stored for about 5 months at 0 °C with 25% R.H. The stigma becomes receptive within 2–3 hours after opening and remains so up to 48 hours thereafter. The maximum fruit set occurs when the stigmas are pollinated within 2 hours after anthesis. Singh and Sehgal (1968) have reported that the receptivity commences even 2 days before anthesis and lasts up to 4 days after anthesis.

However, Singh and Sehgal (1968) obtained maximum germination of pollen grains on stigma when pollination done just 2 hours after anthesis. In wild species of *Psidium*, the best period of stigmatal receptivity is within an hour of opening of the flowers. For making crosses in a hybridisation programme, flowers are emasculated at least one hour before anthesis and bagged. It is pollinated within 2 hours after emasculation with freshly collected or stored pollens and rebagged. Bags are removed only after 5–6 days of pollination. In the beginning of the rainy season, in case irrigation is provided, the new hybrids should be taken to the field (6×4 m), because the most effective evaluations are accomplished after the initial phase of plant development.

3.6 Breeding Progress

Historically guavas have been grown from seed and plantings that are quite variable due to insect pollination of flowers. Seedlings segregate a lot and this has been the basis of the variation used for selection all over the world by breeders as well as growers. Schrader et al. (1954) reported considerable variability among guava seedlings, leading to real possibilities in the selection of quite different genotypes. In conventional improvement, Soubihe Sobrinho (1951), at IAC, developed a scheme for the improvement of guava and determined the fruit set percentage (22%),

indicating predominance of selfing in the species. Later, the same author determined the cross-pollination rate in guava tree (Soubihe Sobrinho and Gurgel 1962), reporting a rate of crossing from 25.7 to 41.3% with an average of 35.6%, the same standard value presented by Nakasone and Paull (1998).

Considerable variability is present whenever a seedling guava population is obtained. Du Preez and Welgemoed (1990) observing plantings of guava seedlings at the CSFRI, Nelspruit, verified production of trees with fruit that varied widely in physical and chemical characters. This variation was used as a means of selecting cultivars to improve stability of an industry based only on one cultivar, namely Fan Retief. From an evaluation of 8,000 trees, 5 selections were made over a 3 year period. Differences were found among seedlings in fruit size, shape, flesh thickness, flesh colour, soluble solids, acidity and ascorbic acid. With the exception of ascorbic acid, all other characteristics were better in the selections than in Fan Retief. The variability observed in these fruit traits indicates that they would be responsive to further, more controlled selection and breeding.

Dinesh and Yadav (1998) provided the analysis of half-sib progenies derived from the variety Apple Colour and verified that the genotypic variability was smaller than the phenotypic for all the studied characteristics. They reported that the level of genetic variability was low and the heritability showed to be moderately high for all of them. Physiochemical characters of the fruits, such as shape, texture, pulp ratio, peel and pulp colour and contents of sugars, acids and volatile compounds plays a significant role in the selection process. Martinez Jr (1992) and Carvalho (1996) verified that pulp colour, soluble solids content and flavour of the fruits were the attributes that most contributed to the rejection of undesirable plants in the programmes of genetic improvement of the guava tree at UNESP (Brazil). Schrader (1955), in his research towards improvement of guava in Brazil, has found genotypes producing fruits of up to 475 g and others with vitamin C indexes of up to 560 mg of ascorbic acid/100 g of fruits. He also considered fruit shape and colour, pulp texture and seed amount.

In some areas of Brazil, use of cultivars with production that does not coincide with the normal pick of the harvest (precocious or late) can provide a crop with a better price in the fresh fruit market (Gonzaga Neto et al. 1991[a]; Gonzaga Neto et al. 1991b; Gerhardt et al. 1995). However, in the State of São Paulo and in other areas, with innovative cultural practices (pruning and irrigation), guava production is extended practically throughout the year.

3.6.1 Breeding for Disease Resistance

A guava tree is attacked by several pests and diseases that harm mainly the fruits (Campacci and Chiba 1983). Rust, caused by *Puccinia psidii* Wint., is one of the most serious diseases of the guava tree, limiting cultivation due to damage to the fruits, spoiling them for consumption as well as for processing (Campacci and Chiba 1983; Figueiredo et al. 1984). Till date, the only feasible control measure is through weekly sprayings with fungicides, which raises production costs.

Wan and Leu (1999) crossed and selfed 12 varieties and lines in 32 combinations and 9,434 resulting seedlings were inoculated artificially with spores of *Myxosporium psidii*, agent of guava tree wilt in Taiwan. One year later, almost half (46.85%) had died due to the disease, a proportion that increased to 98% after 6 years and 192 remained healthy. The commercial variety, Peipa, seems to be the best resistance source, having the largest survival rate (11.69%) obtained among the descendants of the crossing Peipa × R1 (this last one, a lineage). Out of the surviving clones, 57 were selected by their fruit quality, having derived from 8 crossings and one selfing.

Ribeiro and Pommer (2004) studied half-sib progenies resulting from 22,950 seeds from fruits originated through open pollination of 306 accessions. Seedlings were grouped into different number of accessions as: (a) 35 primary selections of white guava, obtained in the breeding programme IAC (identification: White LxPy); (b) 64 primary selections of red guava, obtained in that same programme (identification: Red LxPy); (c) 118 commercial varieties (some with 2 up to 6 accessions) as Supreme, Indiana, Weber, FAO, Australian, Patillo, Paluma, Rica, Ruby Supreme, IAC-4 and others; (d) 55 advanced selections of IAC programme (with acronym MAS), of Conceição de Almeida, BA (with acronym EEFT) and others named Sigla (II to XIII) and; (e) 34 accessions not clearly identified or without identification. Selection was applied in the initial stages of the seedlings and after artificial inoculation with the fungus. The heritability for rust resistance was estimated in a broad sense being $h2 = 0.275$. The results of the evaluations in the half-sib progenies showed variation in the proportion of plants without symptoms: 25% in Group 1 (IAC selections of white guava); 28% in Group 2 (IAC selections of red guava); 44% in Groups 3 (commercial varieties) and 5 (miscellany); and 64% of plants without symptoms in Group 4 (advanced selections of Monte Alegre do Sul and of Conceição do Almeida). The analysis of variance showed that the plants of Group 4 differed from others in that aspect (t test, $p > 0,05$) evidencing the selection pressure made in that sense. After 2 years, 105 individual plants were selected with absolutely no symptoms of the disease and are under selection for other traits, such as yield, fruit characteristics, colour and flavour.

An attempt to overrun a problem with Guava Wilt Disease (GWD) in South Africa was done by Du Preez (2006). Through the use of tissue culture she submitted 30,000 seedlings to a fungal filtrate from the fungus after removing its toxin, using this as a selection agent in the tissue culture, and selected 10 that survived the disease. Those that survived were multiplied in tissue culture and transplanted into pot trials. Out of those 10 selections, she selected 3 rootstocks that seemed to do well in the pot trials. TS-G-1 and TS-G-2 are almost resistant. TS-G-3 was tolerant. Fungus was found in the rootstock, but it did not kill the rootstock. No disease symptoms were observed. None of the rootstocks have ideal fruit quality, but according to the author they do compare favourably with the cultivar that was being used. At the moment, new plantings are being made with the rootstock plants being used as cultivars without grafting. The breeding and selection processes are ever continuing using the same methods in the hope of getting better cultivars that are resistant.

Cell-free filtrates derived from *Penicillium vermoesenii* were used by Vos et al. (1998) to screen 30,000 guava seedlings in vitro. Ten promising selections were made and cloned in tissue culture. Three of the selections exhibited 100% tolerance or resistance to GWD. The major advantage of using this technique to screen for resistance is that the juvenile growth phase of the plants could be maintained. This facilitated the use of nodal and split-nodal cuttings from tissue culture derived rammets instead of the slow, conventional propagation techniques such as air-layering and hardwood cuttings. As a result, 25% of the trees lost to GWD in South Africa have been replaced by trees with tolerant rootstocks within a research period of 5 years.

Studying GWD in South Africa, Schoeman and Vos (1998) verified that six months after inoculation with the fungus, all the Fan Retief plants in the non-grafted experiment inoculated in the stem or in the roots were dead. Except for one plant of selection TS-G2, inoculated in the stem, none of the plants of the other selections showed any symptoms. In the grafted trial, 100% of the Fan Retief plants grafted onto Fan Retief were dead six months after inoculation, while only one plant grafted onto selection TS-G3 showed symptoms. In the field trial, three Fan Retief plants were dead three years after planting while none of the plants of the other selections showed any symptoms. These results indicate that these selections are more resistant to GWD than the commercial Fan Retief cultivar. Selection TS-G3 appears to be tolerant to GWD since the Fan Retief scion was affected by the fungus. Selections TS-G1 and TS-G2 have been used as rootstocks for Fan Retief in commercial plantings in South Africa.

3.6.2 Inheritance in Guava

Compared to other organisms, guava is not an appropriate plant for studies on inheritance. The fact of being a perennial tree, demanding huge areas and labour to cultivate, presenting high heterozigosity and demanding large populations for this type of study ends in very little information on inheritance in guava.

Heritability in the broad sense includes all types of gene action such as dominance, additive and epistasis (Ray 2002). Considerable research effort has gone into estimating the heritability pattern in guava. It has been observed that commercially important traits, such as yield, fruit size, certain types of disease resistance and quality characteristics (Vit. C, acidity, pectin, etc.) are often in the low-heritability category. None of these characters are determined solely by major genes, although basic genes, subject to the modifying effects of polygenes, have been identified for some quality characters like skin colour and acidity. Obovoid shape of the fruit is dominant over round (oblate) and pyriform.

Continuing its original studies on guava breeding in Brazil, Soubihe Sobrinho and Gurgel (1962) observed that red is dominant to white pulp colour. Later on, Subramanyam and Iyer (1992) showed that red colour of pulp is dominant to white and that this character is governed monogenically. A linkage was found between

flesh colour and seed size. It was also observed that the attractive pulp colour and high yields of 'Beaumont' can be transferred to other white sweet cultivars.

Seth (1960) reported varietal cross incompatibility since neither fruit nor seed set was obtained when crosses were made between Behat Coconut X Lucknow-49, S1 X Behat Coconut, Behat Coconut X Apple Colour and Apple Colour X S1. Triploidy and some other genetic factors have been reported to be responsible for female sterility. The variation observed in triploids was possibly due to their independent origin from a different diploid variety. At Coimbatore, in the triploid fruits, a black mass of degenerated ovules was observed in the centre due to less stimulation by placenta during fertilisation. There is a need to further study qualitative and quantitative inheritance in order to assist the guava breeder in interpreting phenotypic values in terms of potential genetic gain.

Dinesh and Yadav (1998) studied the F1 progenies of four crosses among 'Apple Colour' and three other guava varieties. They found that genotypic variance was less than phenotypic variance for all the five characters analysed (fruit weight, length, volume, width and TSS). The coefficient of variation also followed the same trend, implying greater manifestation of these characters. The low genotypic coefficient of variation indicated low degree of genetic variability present in half-sib progenies. The higher phenotypic coefficients of variation imply the greater manifestation of these characters. The coefficients of variation indicated only the variability in different characters and did not indicate the heritable portion. The heritability in narrow sense was observed to be moderately high in fruit length (44.45%) and TSS (42.88%). Heritability was least in fruit width (31.68%). Thus, selection can be practiced to improve the yield characters since these traits are controlled by additive effects. The fruit weight had positive correlation with fruit volume, fruit length and width. However, negative correlation was observed with TSS. This character was negatively correlated with other four characters. The genotypic correlation was higher than phenotypic correlation for all the characters except TSS. This can be attributed to the relative stability of the genotypes. This happens not only when genes governing the traits are similar but environmental factors pertaining to it also have similar effects. Coheritability estimates were moderately high for most of the pairs of characters. The TSS goes down with the selection of big-sized fruits. However, selection of medium-sized fruits would not bring down the TSS.

The proportion of genetic and environmental variances for fruit weight (FW), flesh thickness (FLT), flesh weight (FLW), fruit firmness (FF), seed cavity weight (SCW), total soluble solids (TSS), titratable acidity (TA), juice acidity (pH) and ascorbic acid (AA) in guava were estimated with eight genotypes, four trees per genotype and five fruits per tree for two seasons by Thaipong and Boonprakob (2005) (Table 3.6). Eight clones were randomly selected from the collection of breeding materials. These consisted of six white flesh dessert types ('Klom Salee', 'Yensong', 'Pan Seethong', 'Khao Um-porn', 'Pan Yuk' and 'Nasuan'), one red flesh dessert type ('Philippines') and one pink flesh processing type ('Pijit 12–102'). A high proportion of genotypic variance was found with FW, FLT, FLW, SCW and AA indicating that genetic improvement for these traits through breeding and selection was achievable. Seasonal variance was high for pH, while among fruits within

Table 3.6 Estimated variance values of the fruit characteristics of eight clones

Variance (%)						
Trait	Genotypic	Seasonal	Genotype by season	Trees within genotype	Tree by season	Fruits w. tree
FW	64.5	3.0	2.3	2.4	3.2	24.6
FLT	61.8	0.0	3.2	0.0	4.8	30.2
FLW	65.1	1.6	2.0	1.8	3.9	25.6
FF	4.2	22.5	15.2	0.0	7.4	50.7
SCW	43.6	10.7	7.0	6.3	0.0	32.3
TSS	21.2	26.9	7.4	0.2	2.7	41.6
TA	33.4	20.6	2.9	0.0	2.9	41.2
pH	6.3	61.0	5.3	4.2	4.2	19.0
AA	46.8	10.8	17.0	3.9	0.0	21.5

FW, fruit weight; FLT, flesh thickness; FLW, flesh weight; FF, fruit firmness; SCW, seed cavity weight; TSS, total soluble solids; TA, titratable acidity; pH, juice acidity; AA, ascorbic acid.

tree variance was greatest for FF, TA and TSS. The traits that were high in either season were more difficult to improve genetically.

3.6.3 Ploidy and Breeding in Guava

In a guava orchard with 1,600 plants in Brazil, Soubihe Sobrinho, Pompeu, and Gurgel (1961) have found six that differ greatly from the others in growing habit, leaf structure and low fruitfulness. The fruits presented apple-shape with no distinction from external and internal pulp that formed a mass with few seeds (25 on average). They observed that cells of these plants presented 44 chromosomes instead of 22 confirming them as tetraploid plants.

Subramanyam and Iyer (1993) in their review mentioned that cytological studies made on structure and behaviour of chromosomes in different varieties of *P. guajava* indicated that the meiosis was normal with the formation of 11 bivalents at diakinesis and normal distribution of the chromosomes at later stages. The diploid chromosome number of *P. friedrichsthalianum* was also $2n = 22$. A natural triploid was reported in the genus with somatic chromosome number to be $2n = 33$ as also reported in a seedless variety of *P. guajava*, suggesting that triploidy is the cause of seedlessness in guava.

Ray (2002) reported that in guava, majority of the commercial varieties are diploids ($2n = 22$) while the seedless variety is triploid and shy bearing in nature. To evolve a variety with less seeds and better yield potential, crosses were made between a triploid (Seedless) and diploid (Allahabad Safeda) at IARI, New Delhi. Out of 73 F1 hybrid seedlings, 26 were diploids ($2n$), 9 trisomics ($2n + 1$), 5 double trisomic ($2n + 1 + 1$) and 14 tetrasomics ($2n + 2$). Distinct variation in tree growth habit, and leaf and fruit characters were observed.

The breeding behaviour of aneuploids of guava (*P. guajava* L.) such as trisomic, tetrasomic and higher aneuploids has been studied by Mohammed and Majumder (1974). Reciprocal crosses between aneuploids and diploids indicated less than 100% crossability. The aneuploids when used as male parents crossed

less frequently than as female parents and certain aneuploids crossed more readily than others. Differences were observed in fruit size, fruit weight and seed number in the reciprocal crosses. The extra chromosome was found to be transmitted through both the egg cell and the pollen. However, the frequency of transmission was greater through the egg cell than the pollen. As high as 26% transmission of extra chromosomes were obtained through the egg cell. There was no clear-cut difference between trisomics and higher aneuploids with regard to the frequency of transmission of extra chromosomes.

Sharma and Majumdar (Anonymous 2006) identified a promising aneuploid rootstock for guava and demonstrated its potentials. The results on growth have highlighted the dwarfing effect of the rootstock on cultivar Allahabad Safeda. The tree size reduced significantly and it also showed higher yield potential, with an estimated yield of 28.33 tons of fruits per hectare. The trees were found to be tolerant to guava wilt. It produced fruits of better quality in terms of flesh thickness, vitamin C content, softness of seeds (due to light seed weight) and sweetness compared to Allahabad Safeda on its own roots. The trees grew to a height of 3–4 m, and were ideal for high density planting at a spacing of 3 m by 3 m. The short-statured plants had short internodes and small cup-shaped and lanceolate leaves. The dwarf rootstock is a tetrasomic guava developed by crossing a diploid (Allahabad Safeda) with a triploid (Seedless) variety. It has a wider adaptability, dwarfness and field tolerance to guava wilt.

3.7 Molecular Markers in Breeding

In agriculture, biotechnology has become a routine tool in cell and tissue culture to achieve rapid propagation of plant species; in diagnostics, for detecting plant pests and diseases based on the use of monoclonal antibodies and nucleic acid probe; and in genetic engineering of plant species, to introduce new traits and in aiding conventional plant breeding programmes using molecular markers (FAO 2003).

Morphological data have traditionally been used for variability evaluation. In order to supplement and refine the morphology-based descriptions, enzyme markers were used in a first approach to assess genetic variability (Belaj et al. 2003). Subsequently, DNA-based markers provided a new option for genetic studies and showed significant advantages as compared to morphological and biochemical markers (Sunil 1999; FAO 2003).

DNA markers are becoming increasingly important in a wide range of tasks: construction of genetic linkage maps; comparative mapping analysis; tagging economically important genes; marker-assisted selection and map-based cloning. They also provide genetic information in key areas of germplasm conservation both ex situ and in situ (Karp et al. 1997).

There is a great potential for the application of molecular markers to tropical, subtropical and indeed all perennial fruit crops. For instance, in fruit trees, this activity could be complicated by factors such as self-incompatibility, apomixes,

dioecy, seedlessness, embryo maturity, heterozygosis and long juvenile periods. Consequently, conventional breeding and assessment based on morphological markers could be a difficult and slow process (Moore and Durham 1992).

Although biotechnology is becoming increasingly important in agriculture, the fact that over 50% of the agricultural productivity in the world has been achieved through traditional plant breeding should not be ignored. Although DNA marker technology cannot replace plant breeding, it will certainly facilitate this activity by providing new tools to ease the many problems faced by breeders (Sunil 1999).

3.7.1 Types of Molecular Markers

Molecular markers that reveal polymorphisms at the protein level are known as biochemical markers, whereas DNA markers do it at the DNA level. The former are proteins produced as a result of gene expression that can be separated by electrophoresis to identify the alleles. The most commonly used are isozymes that are variant forms of the same enzyme (Vodenicharova 1989). Protein markers reveal differences in the gene sequence and function as co-dominant markers.

Depending upon how the polymorphism is revealed, DNA-based markers can be classified into two categories: hybridisation-based polymorphisms and PCR-based polymorphisms. Some authors have considered a third category that combine both.. DNA markers can be both dominant and co-dominant (Valadez and Khal 2000).

In guava, different PCR-based techniques have been used to verify DNA quality, to establish fingerprint of individual accessions, to assess the genetic diversity, to construct a genetic linkage map and for tagging economically important genes for marker-assisted selection. These are Random Amplified Polymorphic DNA (RAPD) (Williams et al. 1990), Amplified Fragment Length Polymorphism (AFLP) (Vos et al. 1995), Inverse Sequence-Tagged Repeat (ISTR) (Rohde 1996) and Simple Sequence Repeat (SSR) (Litt and Luty 1989; Tautz 1989; Weber and May 1989).

3.7.2 Applications of Molecular Markers for Guava Breeding

3.7.2.1 DNA Isolation and Purification Protocols

The different applications of molecular markers in agriculture have allowed breeders to increasingly utilize it in breeding programmes. Nevertheless, to ensure a routine use of Molecular Biology, it is a pre-requirement to have protocols that would enable fast DNA isolation and purification. Several methods that make possible direct amplification of plant genomic DNA from leaf, seed and root tissue have been reported (Rehman et al. 2001). Some of these techniques, while rapid, have inherent problems of contaminants, such as polysaccharides, polyphenols and other secondary metabolites, which can inhibit the amplification reaction (Sharma et al. 2000).

When DNA was first isolated from a plant species, problems invariably occurred due to the presence of the contaminants mentioned above. With cell rupture, polyphenols and polysaccharides can make contact with nuclei and other organelles. In their oxidized forms, polyphenols bind to DNA covalently, giving it a brown colour and making it useless for most research applications (Rogstad et al. 2001). Polysaccharides are detected in DNA solution by their viscous, glue-like texture, which difficult the pipetting and also makes DNA no amplifiable by inhibition of the Taq polymerase activity and unrestrictable for endonuclease digestion (Sharma et al. 2002).

For tropical fruit trees, these problems have already been reported (Guillermaut and Marechal-Drovart 1992). In guava, leaves are far sensitive to oxidation, resulting in polyphenol presence. A more serious problem is the extremely high content of polysaccharides that co-precipitate with DNA throughout the standard purification (Ramírez et al. 2004). Several protocols have been performed to obtain good DNA quality and concentration (Prakash et al. 2002; Hernández et al. 2003; Rueda et al. 2003). Also, a variation (Ramírez et al. 2004) of the CTAB method described by Doyle and Doyle (1990) and further purification using NucleoSpin Extract Method (Macherey-Nagel 2002; Fig. 3.4) have been used, providing very good results for molecular applications in this crop.

3.7.2.2 SSR Development from *P. guajava* L.

(GA)n and (GT)n micro-satellite-enriched library was developed to improve the type of molecular markers available for genetic studies and further marker-assisted selection in guava as well as its close related species (Risterucci et al. 2005). To

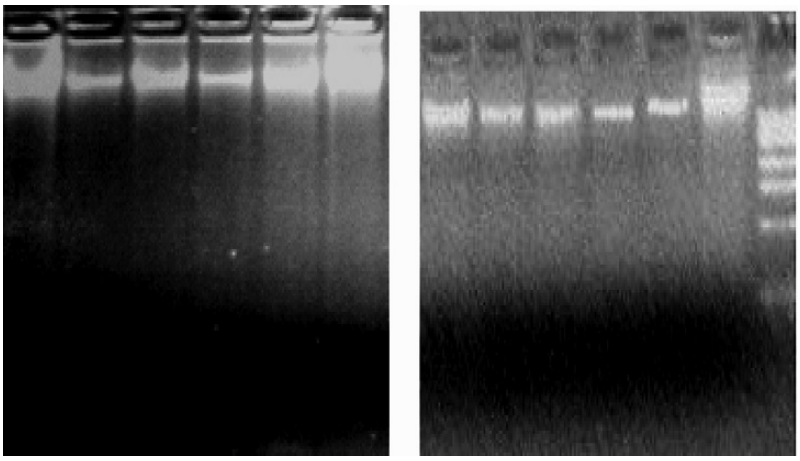

Fig. 3.4 DNA quality and concentration extracted by a modification (Ramírez et al. 2004) of the CTAB method described by Doyle and Doyle (1990) followed by an additional purification with NucleoSpin Extract Method (Macherey-Nagel 2002). Left: Before NucleoSpin purification; Right: After NucleoSpin purification. M: 1 kb DNA ladder marker

determine the usefulness of the primers designed, DNA samples of guava from diverse origins (Cameroon, Colombia, Cuba, Florida, Hawaii and Martinique) and also from *P. acutangulum* D.C., *P. cattleianum* Sabine var. lucidum and *P. friedrichsthalianum* (O. Berg.) Nied. were utilized for PCR amplification. All the SSR primers have been successfully amplified in *P. guajava* L. For the rest of *Psidium* species, except for four loci, the amplification revealed reliable SSR patterns. This library appears to be the first reported for guava and can be used for genotype identification, pedigree analysis, germplasm diversity and mapping studies. Furthermore, it is a potentially useful molecular resource for genetics investigations in the genus *Psidium* (Risterucci et al. 2005). The primer combinations mPgCIR05, mPgCIR07, mPgCIR09, mPgCIR10, mPgCIR11, mPgCIR15, mPgCIR16 and mPgCIR19 revealed clear polymorphism in guava accessions from Cuban germplasm (Rodríguez et al. 2007).

3.7.2.3 DNA Markers for Guava Fingerprinting

The use of molecular markers to establish fingerprint of individual accessions have been suggested for several crops (Sunil 1999). In addition, the International Union for the Protection of New Varieties of Plants (UPOV) is pushing for a distinct, uniform and stable (DUS) testing, the introduction of new test methods and to overcome the legal implications of such changes for plant variety protection (Donini et al. 2000).

In this regard, one approach was the use of four isoenzymatic systems (α-esterase, β-esterase, acid phosphatase and peroxidase) to distinguish intra- and inter-specific variation on *Psidium* spp. However, a clear genotyping was not observed (Albany et al. 1998). Isozymes markers sometimes exhibit an insufficient polymorphism. In addition, spatial-temporal and environment variation could also occur (Dettori and Palombi 2000). Then, their use for identification purposes is restraint to a local germplasm as isoenzyme profiles are not transferable.

With the advent of PCR-based marker system, RADP, AFLP and micro-satellites (SSR) techniques have been the common choice for variety identification in fruit trees (Tessier et al. 1999; Dettori and Palombi 2000; Aranzana et al. 2001; Belaj et al. 2003), but to date, AFLP and micro-satellites are the prevalent option for variety profiling and, hence, identification (Donini et al. 2000). In addition to this, a remarkable degree of polymorphism detected through retrotransposon sequences has also been reported (Inverse Sequence-Tagged Repeat [ISTR]) (Ramírez et al. 2002; Capote et al. 2003).

The selection of a given marker is a balance between the level of polymorphism it can detect (information content) and its capability to identify multiple polymorphisms (Powell et al. 1996). Tessier et al. (1999) defined the D parametre (discriminating power), which evaluates primer efficiency for varieties identification (i.e. the probability that two randomly chosen individuals have different patterns). The D parameter can be used to compare different type of markers even if only the allele frequencies are known.

Despite the fact that a comparative study related with the use of molecular markers for guava fingerprinting is still to be developed, the utility of AFLP, ISTR and SSR for genotype identification have been corroborated (Hernández et al. 2003; Rodríguez et al. 2003; Valdés-Infante et al. 2003; Rodríguez et al. 2007). Besides, RAPD analysis has proven good results (Prakash et al. 2002; Rueda et al. 2003), although their reproducibility across different laboratories remain under discussion (Donini et al. 2000).

3.7.2.4 Genetic Diversity Analysis for Guava Germplasm

A pre-requirement for improving the overall plant characteristics is the knowledge of the structure of the germplasm collection that in turn will lead to a systematic sampling for breeding and conservation purposes. DNA markers have been used to quantify the genetic diversity and determine phylogenetic relationships (Sunil 1999).

Understanding and management of the natural variation present within the domestic cultivars and wild relatives of a plant species is essential for the establishment of an efficient programme aiming at crop improvement. Taking advantage of natural variation is very important for several reasons: genetic uniformity is undesirable because it tends to make the crop vulnerable to epidemics and environmental disasters resulting in yield loss. Many wild relatives contain genes that confer resistance to biotic stresses such as pests and diseases, as well as tolerance to abiotic stresses such as drought, cold and salinity. When such traits are incorporated into economically important varieties, large yield losses can be prevented (Sunil 1999).

Comparisons of molecular markers for measuring genetic diversity have been carried out in several plant species (Belaj et al. 2003), but to our knowledge, such studies have not been so far reported in guava. However, different molecular markers have been used individually to asses the genetic diversity.

Some studies on Myrtaceae have revealed the utility of isozyme to assess the structure and diversity in *Eucalyptus* spp; *Eugenia dysenterica* D. C. and camucamu (*Myrciaria dubia*) (Kunth) McVaugh populations (Turner et al. 2000; Pires de Campos et al. 2001; Teixeira et al. 2004). Although biochemical markers have not been broadly exploited in guava, they can be a potential tool for variability estimation in this species.

Rueda et al. (2003) found a relatively high level of genetic diversity in Corpoica Palmira germplasm (Colombia); while Prakash et al. (2002) detected from low to moderate variability in India germplasm, both using the same molecular marker (RAPD). On the other hand, Valdés-Infante et al. (2003) detected a low level of diversity using AFLP, although micro-satellites revealed a moderate heterozygosis level in the same Cuban guava germplasm (Rodríguez et al. 2007). This difference might result from the inheritance of each molecular marker and the genomic region explored. The codominant nature of SSRs markers allows the detection of a high number of alleles per locus and contributes to detect higher levels of expected heterozygosity than AFLPs. However, this also depends on the species under study (Belaj et al. 2003).

Genetic diversity can be associated with geographical origin of different genotypes within species. In addition, climatic differences in the same region can lead to ecotypes and therefore to new variability sources (Zizumbo-Villauea et al. 2005). Using RAPD markers in guava germplasm collections, some authors have identified genotypes coming from diverse foreign regions (Prakash et al. 2002; Rueda et al. 2003). Nevertheless, overall interpretation of the genetic relationships among guava accessions with AFLP (Valdés-Infante et al. 2003) and SSR (Rodríguez et al. 2007) in Cuba indicates the absence of separate clusters representing local and foreign germplasm. This reflects the selection of guava lines from open pollination rather than from controlled crosses. Micro-satellites also detected a high number of alleles shared for the majority of guava genotypes in this germplasm. This suggests that most of the plant material analysed shares a common genetic ancestry; this comes from the fact that relatively few accessions were used for breeding programmes and many hybrids derived from them were conserved in the germplasm bank. On the other hand, some individual and combined rare alleles were detected in such accessions. This information provide ground for parental selection in guava breeding programmes and conservation strategies.

The correspondence among the results derived from individual data sets is by far the most important issue to be considered when combining different data sets. Several studies in recent years have analysed correlation among genetic distance-similarity matrices derived from the application of various DNA-based marker systems. However, very few analyses have attempted to compare results originated from individual versus combined data, regarding the genetic diversity assessment and collection management (Mohammadi and Prasanna 2003).

The generation of a higher number of polymorphic markers is not necessarily correlated with the resolution power (Capote et al. 2003). Although AFLP detected lower diversity than SSR in the guava collection from Cuba, the second could not identify between two highly related genotypes ‘N6’ and ‘Ibarra’, using the same primer number (Rodríguez et al. 2007). This corroborates the necessity of an integrated study to prevent wrong deductions related to misclassification, duplicates detection and variability estimation.

Also, a comprehensive analysis of data set of distinct nature (morphological, biochemical and DNA markers) to ascertain whether such combination provides a better understanding of genetic diversity is highly scarce (Mohammadi and Prasanna 2003). The use of different methods to evaluate genetic diversity may reveal dissimilar patterns of variation. Phenotypic differences are not necessarily correlated with the number of underlying gene mutations and differences in phenotypic characters are not necessarily reflections of different genetic events (Persson 2001). Besides, morphological traits are often influenced by environmental conditions. On the other hand, DNA markers can cover coding as well as non-coding regions of the genome. For that reason, classical methods for evaluating genetic variation have been complemented by molecular techniques (Persson 2001). The low correlation coefficient detected by Rodríguez et al. (2004) from the comparison of similarity matrixes between morph-agronomical and AFLP analysis in guava germplasm corroborates this assertion.

3.7.2.5 Construction of Guava Genetic Linkage Map

In order to efficiently use the countless polymorphisms as genetic markers, knowledge of their individual genomic locations is necessary and this information can be obtained by constructing a genetic linkage map. Thus, a genetic linkage map graphically represents the arrangement of the innumerable loci, which includes morphological and isozyme as well as DNA markers along with the chromosome (Sunil 1999).

For a future implementation of marker-assisted selection to improve the efficiency of guava breeding programme in Cuba, three mapping populations were produced under controlled-pollination conditions with three individual trees of the cultivar 'Enana Roja Cubana' as the female parent and pollen from cultivars 'N6' (mapping population 1 = MP1), 'Suprema Roja' (MP2) and 'Belic L-207' (MP3).

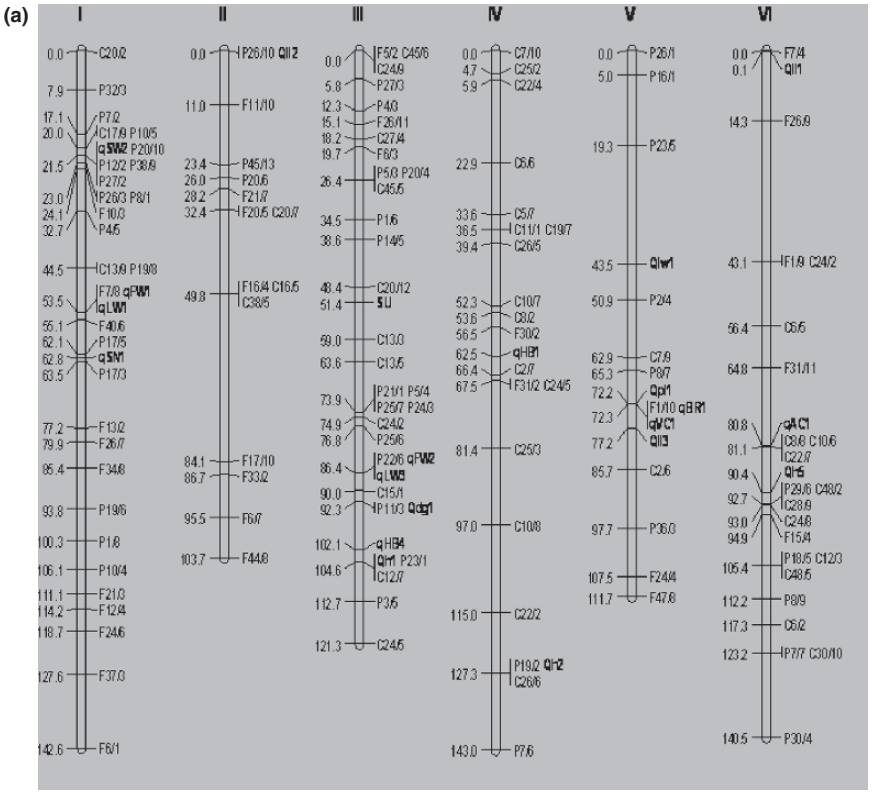

Fig. 3.5 **a** and **b**: Integrated molecular linkage map for guava mapping population MP1 (Rodríguez et al. 2007). Single linkage groups are indicated in roman numbers with distances on the left given in cM. Mapped AFLP markers are listed by their origin as to the parent (P: parent 1, 'Enana Roja Cubana'; F: parent 2, 'N6'; C: marker common to both parents) and the AFLP primer combinations (e.g. P17/3 is the parent 1-specific AFLP fragment #3 produced by primer combination 17). The mapped QTLs are indicated in bold

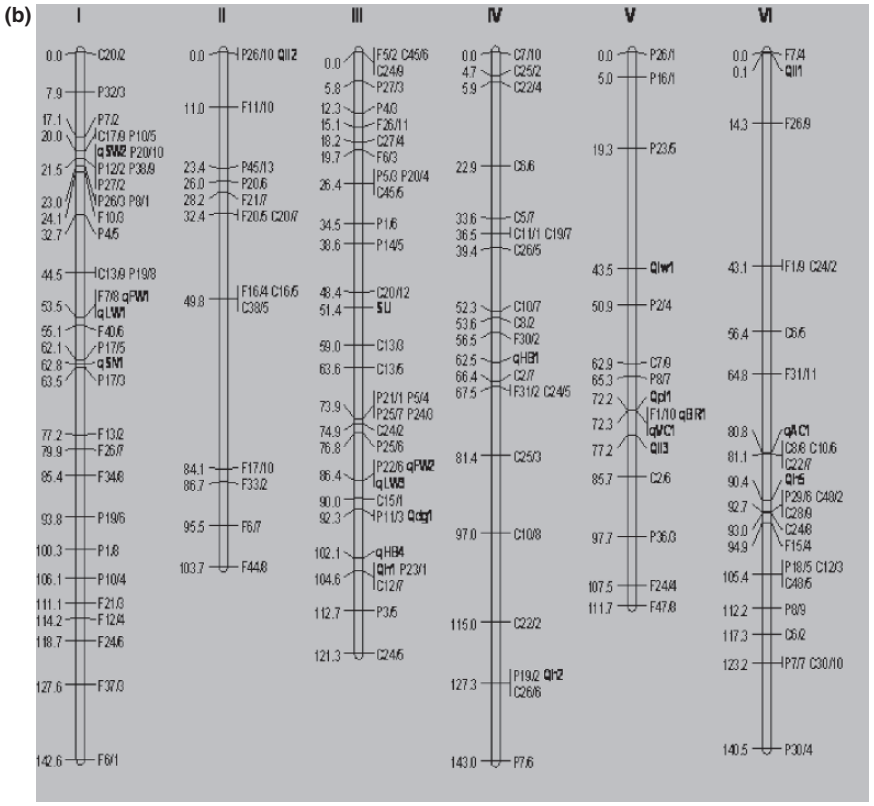

Fig. 3.5 (continued)

The MP1 was used for the initial identification of co-segregant markers in the progeny by AFLP technique.

Based on these results, the first genetic linkage map was established by Valdés-Infante et al. (2003) with a total of 167 markers mapped onto 11 linkage groups that presumably represent the 11 chromosomes of the haploid guava genome. These efforts were further extended by increasing the number of AFLP primer combinations and mapping additional markers onto the linkage map. The high number of common markers enabled the fusion of the two individual parental maps into an integrated linkage map for the two guava genotypes with a total of 220 markers mapped up to date (Fig. 3.5). The individual linkage groups contain from 11 to 30 markers each, vary in length between 104 and 150 cm, and result in a total map length of 1,379 cm (Rodríguez et al. 2007).

Although the initial investigations about genetic linkage maps have been developed on cereals species, this technique can represent an important and efficient tool for fruit trees breeding programme; due to long juvenile periods that delay the evaluation and characterisation of fruit and overall plant for many years (Moore and Durham, 1992; Kijas et al. 1997).

3.7.2.6 Marker-Assisted Selection in Guava

A direct application of genetic linkage maps has been in tagging genes of economic importance with molecular markers (Mohan et al. 1997). In general, the likelihood of identifying a marker linked to a gene is inversely proportional to the distance between the marker and the gene. Several important traits such as yield, fruit quality and maturity, and resistance to several biotic and abiotic stresses are controlled by a relatively large number of loci, each of which makes a small positive or negative contribution to the final phenotypic value of the trait. Such loci are termed 'quantitative trait loci' (QTLs) and those traits that show a continuous variation in phenotype are termed 'polygenic traits' because the final phenotypic expression is determined by the genetic variation at a large number of loci, modified by environmental effects (Sunil 1999).

By using molecular markers, chromosomal positions can be assigned to individual QTLs in order to establish the types and magnitude of gene effects of individual QTLs and also to determine which parent possesses the positive allele at each QTL (Sunil 1999).

Morphoagronomic characters such as leaf length, leaf width, petiole length, height and the growth rates for height and trunk diameter were recorded at regular intervals on the established guava mapping population (MP1; see 3.7.2.5 topic). Fifteen QTL loci, which originated predominantly from 'N6' (male parent of MP1; tall genotype in contrast to the female dwarf 'Enana Roja cubana') could be mapped in total (Valdés-Infante et al. 2003).

Subsequently, further characters related to fruit quality (fruit width and weight, seed number and seed weight, total soluble solids, acidity, vitamin C content and pulp thickness) were also assessed. In total, 21 QTLs were identified for these traits and mapped onto different linkage groups of the integrated linkage map (Fig. 3.5). Additional efforts along these lines will form the basis for marker-assisted selection (MAS) in guava breeding programme (Rodríguez et al. 2007).

3.7.3 New Trends

The results presented here are a further step (1) to compare the discriminating capacity and informativeness of the different molecular markers for genotype identification and genetic diversity analyses; (2) to determine the genetic similarity estimates and genetic relationships among genotypes as well as to compare the patterns of variability between morph-agronomic and molecular markers; (3) to characterise wild relatives looking for germplasm diversity and resistance to biotic and abiotic stresses; (4) to increase the marker density of the guava molecular linkage map; (5) to identify co-dominant DNA marker such as micro-satellites for an alignment of individual maps into a guava reference map; (6) to identify markers that co-segregate with important breeding traits; (7) to detect resistance gene-like sequences (RGLs) as potential candidates for resistance genes to map these RGLs

onto the guava map and record a putative segregation of tolerance in the mapping population to pest and diseases.

3.8 New Varieties: Present and Future

Research institutions from countries where guava is a cash crop, such as India, Brazil, Mexico and others devote substantial efforts to produce and release new varieties. The example of India, where guava improvement work for the first time was initiated during 1907 at Ganeshkhind Fruit Experimental Station, must be followed by others. Subramanyam and Iyer (1993) listed a number of promising hybrids from different Research Stations in India, resulting from a survey of many authors and their own work.

At Narendra Dev University of Agriculture and Technology, Faizabad, out of the 23 strains collected as a result of survey in guava growing region, 3 seedlings of Allahabad Safeda (AS1, AS2 and AS3) and 2 of Faizabad Selection (FS1 and FS2) were found to be promising with respect to fruit quality and yield. From plantations around Navalur, a village in Karnataka, 16 high performing seedlings were selected from the variety Navalur, which is hardy, drought tolerant and canker resistant, based on fruit quality, yield and plant characters. Twelve strains were collected from Aurangabad and Bhir districts of Marathwada, out of which ABO 3 and BHR 3 and 5 were observed to be superior. At IIHR, Bangalore, from 200 open pollinated seedlings of variety Allahabad Safeda, one seedling selection, Selection-8, was found to be promising. Plants are dwarf and give higher yields.

At IIHR, Bangalore, by hybridisation among Allahabad Safeda, RedFlesh, Chittidar, Apple Colour, Lucknow-49 and Benaras, 600 F1 progenies were raised. Two selections, Hybrid I and Hybrid 16-1 were found to be promising. Hybrid-I: hybrid between Seedless and Allahabad Safeda, giving heavy yield. Fruit size is medium, pulp is white with few soft seeds. Fruit quality in terms of sugar content and TSS is excellent. Keeping quality is good. Hybrid 16-1: from a cross between Apple Colour and Allahabad Safeda, showing plants semi-vigorous plants giving a moderate yield. The fruit skin has a very attractive bright red colour. The flesh is firm, white with very high TSS and good keeping quality. The seeds are few and soft.

At Horticultural Experiment and Training Centre, Basti, inter-varietal hybridisation was undertaken to obtain a variety with higher vitamin C and attractive skin and flesh colour utilizing the cultivars Allahabad Safeda, Seedless, Lucknow-49, Patilla, Apple Colour, Kothrud and Red Flesh. Fifty-five F1 hybrid seedlings were obtained by crossing which are being evaluated. At Fruit Research Station, Sangareddy (AP), inter-varietal hybridisation resulted in the isolation of two superior hybrids, Safed Jam and Kohir Safeda, which were released for commercial cultivation, particularly in semi-arid tropical areas of Telangana and Rayalseema. Safed Jam: a hybrid between Allahabad Safeda and Kohir, is similar to Allahabad Safeda in growth habit and fruit quality. The fruits are bigger in size with good quality and few soft seeds.

Kohir Safeda: it is a heavy yielding cross of selected line of Kohir x Allahabad Safeda. Tree is vigorous, the fruits are larger with few soft seeds and white flesh.

Ray (2002) described 8 guava cultivars recently developed through selection, such as Allahabad Surkha, Lalit and Bangalore local and other 12 superior guava hybrids developed at different fruit research centres in India, such as Safed Jam and Kohir Safeda. One hybrid 'Arka Amulya' has already been released and two (Hybrid 16-1 and Hybrid 31-1) are likely to be released shortly on account of their better characteristics.

Pereira and Nachtigal (2002) in Brazil, at UNESP, Jaboticabal, started in 1976 a selection programme of new cultivars, through the introduction and selection of seedlings originated from open pollinated American, Indian and Brazilian varieties of different provenance and local selections, leading to the obtaining of the cultivars Rica and Paluma (Fig. 3.6). Since 1985, in that same institution, the second phase of genetic improvement of the guava tree is under development, with the goal of obtaining plants with favourable agronomic attributes and fruits that can be destined to industrialisation as well as for consumption as fresh fruit. After a long evaluation and selection period, the programme achieved selections with potential as new options for the guava growers. The following crossings were accomplished:

8501 – Rica × EEF-3
8502 – Supreme-2 × Paluma
8503 – Rica × Patillo 5
8504 – Paluma × Rica.

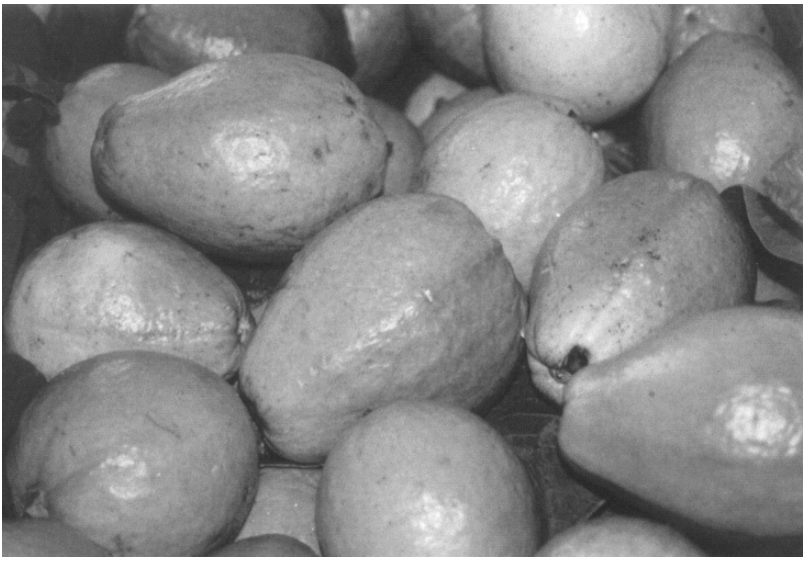

Fig. 3.6 Paluma, the guava cultivar most planted in Brazil
(Source: TodaFruta (www.todafruta.com.br))

The following genotypes were selected:

8501-01 – It presents productive and vigorous plants and normal maturation period; the fruits are of medium size (122 g without thinning), ovoid, with neck of reduced size, thickness of pulp of 118 mm and around 74%; the pulp is of rosy colour, with soluble solids of 8.4° Brix, relationship TSS/TA 18 and vitamin C content of 134.25 mg ascorbic acid.100 g of pulp-1. The main characteristic of this selection is low susceptibility to the psyllidium (insect, Psyllidae) attack.

8502-01 – Productive plants, with ramifications predominantly horizontal, medium vigour and precocious maturation (about 130 days from blossom to the maturation of the fruits); it presents fruits of big size (193 g without thinning), with a ratio of firm pulp of 76%, thick pulp (137.5 mm), ovoid, with neck of reduced size; the pulp is of rosy colour, intense and brilliant, with total soluble solids content close to 10° Brix, relationship TSS/TA close to 20 and vitamin C content around 100 mg of ascorbic acid.100 g of pulp-l; presents few seeds and reduced size.

8503-08 – Presents productive and vigorous plants, and with precocious maturation period; the fruits are of medium size (127 g without thinning), ovoid, with neck of reduced size, thickness of pulp of 113 mm and ratio of firm pulp around 72%; the

Fig. 3.7 Século XXI (XXI Century), a new guava cultivar in Brazil (Source: TodaFruta (www.todafruta.com.br))

pulp is of rosy colour, with soluble solids content of 8.8°Brix, relationship TSS/TA 17 and vitamin C content of 101.45 mg ascorbic acid.100 g of pulp-l. The main characteristic of this selection is the production of fruits lacking a strong pungent odor, even when ripe, characterizing most guava cultivars.

In Brazil, Pereira, Carvalho and Nachtigal (2003) released the XXI Century (Fig. 3.7) guava cultivar obtained from 219 plants originated from several crossings after 10 years of evaluation. It was from the cross Supreme-2 × Paluma and its main characteristics are a very productive plant with a short cycle (130 days from bloom to harvest), big fruits (236 g in average) with thick pulp, rosy-red, great flavour and with little and small seeds.

At least in Brazil, it is quite evident that increases in the area planted with guava and the high yields obtained in the main producing regions are due to the availability of improved varieties, such as Paluma.

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Chapter 4
Breeding Papaya (*Carica papaya* L.)

Ying-Kwok Chan

4.1 Introduction

Papaya (*Carica papaya* L.) is a popular fruit native to tropical America. It is usually grown for its small to large melon-like fruit. It is a herbaceous, short-lived, perennial, bearing fruits continuously at the leaf axils spirally arranged along the single erect trunk. Papaya trees can normally live for 5–10 years, but in commercial plantings they are replanted every 2–3 years because the trees become too tall for economic harvesting. The papaya is also called papaw, pawpaw, papayer (French), melonenbaum (German), lechosa (Spanish), mamao, mamoeiro (Portuguese), mugua (Chinese) and betik (Malaysian, Indonesian).

The papaya is popular as a backyard tree in many developing countries but increasingly becoming more important in commercial plantings for domestic markets and for export in countries like Mexico and Malaysia. The advantage in papaya cultivation is the rapid return of investment due to its early maturation, intensive cultivation and high yield. Most papayas in the tropics can be harvested 8 or 9 months after sowing and yields can range from 60 to 100 t/ha/year for improved varieties. The ripe fruit has a delicate aroma and sweetness and has high contents of vitamins A and C. One medium-sized papaya exceeds the Dietary Reference Intakes (DRI) of 3000 IU for vitamin A and 90 mg for vitamin C, established by the U.S. Food and Nutrition Board (OECD 2004). There is great diversity in the size, shape and quality of the fruit. In unselected germplasm or backyard trees, fruits are usually very large and not very palatable, but varieties such as 'Solo' and 'Eksotika', specifically selected for export or up-markets, are usually small for convenience in packaging and have much better taste and storage attributes.

Papaya is usually eaten fully ripe when the flesh is soft and succulent. However, it can also be eaten raw, sliced into thin strips and eaten as vegetable or processed into various products such as candy, pickle or puree. The 'Eksotika' papayas imported by China are served as a delicacy in high-end restaurants: the half-cut fruit with

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seed scooped out is filled with ‘sharks-fin’ or ‘birds-nest’ and steamed before serving. The latex from unripe fruit and leaves contains a proteolytic enzyme papain, which can be used for tenderizing meat, chill-proofing beer, tanning leather and for making chewing gum. In pharmaceutics, papain is used for suppression of inflammation, treatment of gangrenous wounds and for various digestive ailments. As a proteolytic enzyme, it has exfoliating property that removes the dead surface cells of the skin, giving it a rejuvenated feeling. It is therefore popularly used in soaps, creams, shampoos and lotions in the cosmetic industry.

4.2 Origin, Distribution and Trade

The cultivated species of *C. papaya* L. has not been found wild in nature. Its origin is rather uncertain, but there is some agreement among botanists that it originated in the lowlands of Central America between southern Mexico and Nicaragua (Storey 1969a). It is believed to have originated from hybridisation between two Mexican species. Early distribution over wide geographical regions was enhanced by abundance of seeds in the fruit and their long viability. Following the discovery of the New World, papaya was taken by early maritime explorers along tropical trade routes and reached Panama as early as 1535, Puerto Rico by 1540 and Cuba soon thereafter (Storey 1969a). By 1611, it was grown in India and by 1800 was widely distributed in the South Pacific, Malacca and Philippines. Don Francisco Marin, a Spanish explorer and horticulturist, is credited with the introduction of papaya into Hawaii from the Marquesas Islands during the early 1800s. Papaya is now grown in all tropical countries and in many subtropical regions of the world.

Major commercial production of papaya is found primarily between 23°N and S latitudes. Man has extended cultivation into regions as far as 32°N and S latitudes. At these latitudes, papayas may be best grown in well-protected areas at sea level or in greenhouses as seen in the Canary Islands and Okinawa. In Hawaii, at 19–22°N, papaya is grown at sea level and up to 300 m elevation (Nakasone and Paull 1998).

The global production of papaya in 2004 was 6.75 million t (Anon. 2006). Of this, 24% was produced by Brazil (1.65 million t), followed by Mexico, Nigeria, India and Indonesia, each contributing 9–14% of the world production. The most important papaya exporter in 2004 was Mexico at 96,525 t or 34.8% of the world export, followed by Malaysia (21.0%), Brazil (13.0%), Belize (10.4%) and USA (Hawaii) (3.5%). The most important importer of papaya was clearly USA with 126,024 t or 47.6% of the global import. Undoubtedly, papaya’s appearance in the American market was from Mexico and Hawaii. The next largest importers were China and Singapore with 11.9% and 9.3% respectively of the total world import. The major supplier to these markets is Malaysia. The papaya imported by the European markets like Netherlands (5.8%) and U.K. (4.2%) are supplied mainly by Brazil and the African nations.

4.3 Ecology

4.3.1 Soil

Papayas are not very fastidious to soil types but they must be well-drained as even short periods of flooding (>8 hours) can lead to root rot and death of plants. A porous loam or sandy loam soil with pH between 5.0 and 7.0 is ideal. At pH levels below 5.0, plant growth is poor and field mortality high and lime applications are needed to increase growth and yield.

Papaya can be grown successfully in certain marginal soils, such as peat, sandy (tin-tailings) and acid sulphate soils, if their inherent shortcomings are taken care of. For peat and acid sulphate soils, high rates of lime application (6–8 t/ha) are essential for successful cultivation. On peat, micro-nutrients like boron, zinc and copper should also be applied regularly to ensure production of quality fruits. Water table in peat and acid sulphate areas is usually high and the area requires drainage before papaya, which is very sensitive to flooding, can be grown. Yields of more than 100 t/ha for some selected hybrids have been obtained from peat soils (Chan and Raveendranathan 2003). However, one major constraint encountered on peat is the poor root anchorage and trees will lodge if they are not staked or supported. It is also not possible to employ heavy farm equipments on the soft peat ground.

Cultivation of papaya on sandy soils requires incorporation of large amounts of organic matter such as chicken dung and Palm Oil Mill Effluent (POME) (>30 t/ha), heavy chemical fertilisation (8 kg/plant/year) coupled with irrigation facilities. Susceptibilities to boron deficiency and nematode infestation are two major problems encountered on sandy soils.

Papaya has been ranked from extremely sensitive to moderately tolerant to salinity. Germination and early seedling growth appear to be the most sensitive stages. It is probably moderately salt sensitive at other growth stages.

Papayas cultivated on marginal soils have a relatively shorter economic life span; the texture and sweetness of the fruits may be slightly inferior to that obtained from the usual loamy soils.

4.3.2 Climate

4.3.2.1 Rainfall

Papaya is a continuous cropper and fruiting may be disrupted even with short periods of drought lasting a week or so. It grows and yields well in the humid tropics where there is a uniform annual precipitation of about 1,200 mm. However, supplementary irrigation is still needed as perfect uniform rainfall distribution does not occur in tropical areas with monsoon-type climates of well-defined wet and dry seasons. Irrigation can increase yield by 20% over rain fed papayas in Malaysia and this increase more than compensates for the cost of the irrigation system (Chan et al. 1991).

During drought season, water stress causes flowers to go into a sterile phase resulting in non-productive 'skips' in fruit production. During the wet season, the consistently high moisture level promotes the production of hermaphrodite flowers with reduced number of stamens (Awada 1961). Such flowers develop into misshapen or 'cat-faced' (carpelloid) fruit that are unmarketable. Drought also leads to the rapid shedding of older leaves and the fruit stay unripe on the trees longer. With the sudden onset of rain, several fruits may be harvested from the same tree as they simultaneously turn 'breaker-colour'. Prolonged wet spells, especially in poorly drained areas, are detrimental to papayas. Within five days of continuous rainfall, chlorosis of the lower leaves is evident and in severe cases, the leaves lose their turgidity and hang bunched around the trunk but do not abscise. Recovery from non-lethal flooding is slow due possibly to the low regeneration of new roots in fruiting trees. In Queensland, Australia, a dieback disorder of papaya was linked to excessive rainfall followed by a hot, dry period and exacerbated by heavy, poorly drained soils (Glennie and Chapman 1976)

4.3.2.2 Temperature

The optimum temperature for papaya growth is between 21 °C and 33 °C. Papaya is extremely sensitive to frost and if temperature falls below 12 to 14 °C for several hours at night, growth and production are severely affected (Nakasone and Paull 1998). Dioecious cultivars are more stable towards variation in temperatures, as female trees at <20 °C do not exhibit the sex changes shown by the more sensitive hermaphrodite cultivars. Cool temperatures cause a reduction in stamen number in hermaphrodite flowers, a condition commonly known as 'carpellody'. Hermaphroditic cultivars like Solo grown under minimum temperature, less than 17 °C, can have 100% carpelloid flowers. At higher temperatures (>35 °C), there is a tendency of hermaphrodites to form functional male flowers with poorly developed and non-functional female parts. Reversal to this sterile stage leads to skips in fruiting. This tendency varies with cultivars as well as within a cultivar. Net photosynthetic rate also rapidly declines above 30 °C. High temperature following excessive moisture was believed to cause the dieback disorder of papaya (Glennie and Chapman 1976).

Temperature during the growing season significantly influences fruit development and maturation and may stretch it from the normal 120 to 150 days, especially in subtropical areas. Flowers and fruit usually abort in winter and if the fruit is set in late fall, it can take up to 90 days longer to mature. Such fruits are unattractive, with pale flesh colour and low total soluble solids. Final fruit size is determined in the first 4–6 weeks of fruit development and temperature plays a dominant role in the process, especially in subtropical areas (Nakasone and Paull 1998).

4.3.2.3 Radiation

Papaya is a sun-loving plant. When shaded, the plants are etiolated, have smaller leaves, lower stomatal density, increased inter-node and petiole length and decreased

chlorophyll content. Partial stomatal closure and opening occurs rapidly with cloud related changes in irradiance, thereby maximizing plant/water use efficiency (Clemente and Marler 1996).

No photoperiodic effects on tree growth, production or sex expression were reported (Lange 1961). Allan, McChlery and Biggs (1987), however, reported that short daylengths coupled with cool temperature caused reversion of usually sterile staminate flowers from male trees to fertile, elongate type hermaphrodite flowers.

4.3.2.4 Wind

Papaya trees are delicate and require protection from strong winds. The root system is well-developed but relatively shallow and the tree can be uprooted by winds of $64\,km\,hr^{-1}$, especially if the soil is softened by rain (Nakasone and Paull 1998). Even though trees may withstand uprooting, considerable shattering of large leaves occurs, resulting in reduced photosynthesis. This leads to flower and young fruit abscission and reduction in total soluble solids of the maturing fruit. Abrasions of the fruit caused by swaying petioles and leaves also scar the fruit and reduce its marketability. Recovery from wind damage can take from 4 to 8 weeks.

4.4 Taxonomy and Species Relationship

Papaya belongs to the family Caricaceae under the genus *Carica*. Until recently, this family consisted of three other genera, namely *Cyclimorpha*, *Jacaratia* and *Jarilla* and *Carica* itself was made up of 21 species (Badillo 1993). However, the family Caricaceae is now re-classified as having six genera (with the addition of *Vasconcellea* and *Horovitzia*) and *Carica* now has only one species, namely *C. papaya* that represents the cultivated papaya (Badillo 2000). *Vasconcellea*, whose species were formerly assigned to the genus *Carica*, includes several species with edible fruits and have great importance for breeding and genetic studies. The species with edible fruits are *V. cundinamarcensis* (chamburo), *V. pubescens* (ababai), *V. stipulata* (siglalon or jigacho), *V. monoica* (col de monte), *V. goudotiana* (papayuelo), *V. quercifolia* and *V. cauliflora* (bonete or mountain pawpaw). Most of the fruits from *Vasconcellea* are small with thin, dry pulp and are usually cooked and flavoured before eating. *V. pentagona* (babaco) is of subtropical origin found in the highlands of Ecuador (Oosten 1986) and commercially cultivated for its large, parthenocarpic fruit in New Zealand (Little 1982).

Some *Vasconcellea* species have resistance to diseases which *C. papaya* is susceptible to, for example *V. cauliflora* and *V. quercifolia* are resistant to distortion ringspot virus and some attempts have been made to transfer resistance to *C. papaya* by inter-generic hybridisation. Successful inter-specific hybrids have been reported among *Vasconcellea* species but *C. papaya* was not cross compatible with any of them (Mekako and Nakasone 1975). However, hybrids of *C. papaya* with *V. cauliflora* (Manshardt and Wenslaff 1989a) and with *V. pubescens, V. quercifolia* and *V. stipulata* (Manshardt and Wenslaff 1989b) were obtained using embryo

rescue techniques to overcome post-zygotic barriers to hybridisation. More recently, Drew, Siar, O'Brien and Sajise (2005) reported success in inter-specific hybridisation between *C. papaya* and *V. quercifolia* and a few inter-specific hybrids resistant to papaya ringspot virus have been selected. These back-crossed fairly easily to *C. papaya* and further back-crosses would be expected to develop a resistant genotype with the economic traits of *C. papaya*.

4.5 Mating System

C. papaya is a polygamous species. In nature, it is dioecious with male and female trees in the population, but possibly due to man's interference and deliberate selection against non-productive male trees, gynodioecious populations with female and hermaphrodite trees also exist (Storey 1969a).

For dioecious populations, several workers (Prest 1955; Storey 1969a) agreed that wind is the main agent of pollen dispersal. The long pendulous male inflorescence, which readily sheds pollen in the breeze, lends support to this belief. However, Allan (1963) reported that very little papaya pollen was airborne and suggested that honeybees were responsible, although papaya flowers were not the priority sites for visits. Free (1975) reported that papaya flowers were often visited by many Skipper butterflies (*Perichares philetes philetes*) during dusk in Jamaica and suggested their use as pollinators if the need arose. Garret (1995) showed that pollination by wind and honeybees was rare in Queensland, Australia; the main pollinator being hawkmoth (*Hyles* sp.). Some native vegetation in Queensland and cultivation of grape and sweet potato help to attract these pollinators and increase fruit production in papaya. Dioecious papaya varieties are enforced cross-pollinators because of physical separation of the androecium and gynoecium.

In gynodioecious populations, the role of wind as the pollinating agent is diminished. This is because the stamens are packed inside the corolla tube and seldom protrude prominently out of the flower. Many gynodioecious varieties such as 'Sunrise Solo', 'Kapoho Solo' and 'Eksotika' are self-pollinated and are, therefore, purelines. The hermaphrodite flowers are mostly cleistogamous, namely, anthers dehisce and release the pollen to effect self-pollination prior to anthesis of the flower (Rodriquez-Pastor et al. 1990). Such varieties are enforced self-pollinators and the seed gathered from this hermaphrodite fruit will usually breed true to type. Self-pollination in papaya does not appear to result in any loss of vigour (Hamilton 1954).

Parthenocarpic seedless fruit may sometimes form without fertilisation of flower. This usually happens to isolated female trees without any nearby pollen source. Parthenocarpy is usually not a criterion pursued in breeding programme because seeds are not an inconvenience to remove in papaya. Moreover, parthenocarpic fruit are small and although seedless, the tiny undeveloped embryos are bitter and still need to be scraped off before the fruit is eaten.

Papaya pollen production and viability varies according to variety and season. In the production of the F_1 hybrid 'Eksotika II', two inbred parents Line 19 and Line 20 were used, with the latter as male parent because of consistently higher

pollen production (Chan 1993). Variation in pollen production was observed with decreased quantities during winter and early spring (Garret 1995). Pollen from freshly anthesized stamens have 90% viability during summer, but drops to as low as 4.5% in winter when temperatures below 10 °C severely affect viability because of degeneration of pollen mother cells (Allan 1963). Under ideal storage conditions, pollen remains viable for 5–6 years. In contrast to pollen production and viability, stigma receptivity remains high throughout the year. Both female and hermaphrodite flowers pollinated with viable pollen will successfully set fruit even in winter.

In studies of pollen movement using GUS transgene marker, Manshardt et al. (2005) have shown that papaya trees located 0.4 km downwind, did not get pollinated by pollen from acre of GUS-marked 'Rainbow' papaya. The Papaya Biotechnology Network of Southeast Asia (ISAAA) has also proposed a separation of 400 m as safe distance for preventing gene transfer from transgenic to non-transgenic papayas (Hautea et al. 1999). Singh (1990) recommended 2–3 km isolation distance to preclude foreign pollen from contaminating the production of foundation seed for papaya.

4.6 General Biology

4.6.1 Stem

The stem is soft-wooded, usually single and erect and sometimes branched if the terminal shoot is injured. The stem is hollow and marked by prominent half-moon shaped leaf scars on the surface. A 2-year-old tree in an orchard will have a trunk diameter of 20–25 cm at the base and about 5–10 cm near the crown of leaves. The height would be about 4–5 m.

4.6.2 Leaves

The leaves are clustered at the apex of the stem. They consist of large, palmate laminae, 40–60 cm in width, normally with 7–9 lobes and held by long, hollow, pale green or purple tinged petioles. Usually 15 mature leaves are present; the older, lower leaves shrivel and sometimes remain attached for a while to the trunk. Under Malaysian conditions, varieties like 'Sunrise Solo' and 'Subang 6' produce about three leaves per week (Chan and Toh 1984).

4.6.3 Fruit

The papaya fruit is a fleshy berry, variable in weight from 200 g to well over 10 kg. Fruit shape is a sex-linked character and ranges from spherical to ovoid from female flowers to long, cylindrical or pyriform (pear-shaped) from hermaphrodite flowers. The skin of the fruit is thin and usually green when immature, turning to yellow or

orange when ripe. The immature fruit, when bruised, exudes a white sticky latex that contains a proteolytic enzyme papain. The flesh is succulent, usually yellow or reddish orange in colour. The fruit has a central ovarian cavity that is lined with the placenta carrying numerous black seeds. The ovarian cavity is larger in female fruit than in hermaphrodite fruit. The shape of the cavity at the transverse cut ranges from star-shaped with 5–7 furrows to smooth circular wall.

4.6.4 Seed

The papaya seed consists of a small laterally flattened embryo with ovoid cotyledons surrounded by fleshy endosperm and a seed coat made up of a dark brown, hard, muricate endotesta and a translucent sarcotesta that contains a thin mucilaginous fluid. This fluid contains growth inhibitors that prevent germination while the seeds are still in the fruit. A well-pollinated fruit produces about 800–1,000 seeds attached to the interior wall (placenta) of the ovarian cavity.

4.6.5 Floral Biology

4.6.5.1 Flower Types and Sexes

Storey (1941) classified papaya flowers into five basic types:

Type I: Pistillate or female flower devoid of stamens, with a distinct ovoid ovary terminating in a five-lobed stigma (Fig. 4.1a)

Type II: Hermaphrodite (pentandria) flower having five functional stamens and a globose five-furrowed ovary (Fig. 4.1b)

Type III: Hermaphrodite (carpelloid) flower having six to nine functional stamens and an irregularly ridged ovary (Fig. 4.1c)

Type IV: Hermaphrodite (elongata) flower having ten functional stamens and an elongated, smooth ovary (Fig. 4.1d)

Type IV+: Hermaphrodite (barren) flower having ten functional stamens but the pistil aborts, becomes vestigial and lacks a stigma (Fig. 4.1e)

Type V: Staminate flower having ten functional stamens only. The ovary is completely absent and flowers are bunched in an inflorescence (Fig. 4.1f)

Although five basic floral types are listed, certain male and hermaphrodite trees undergo sex reversal and morphological changes to varying degrees under the influence of climatic and environmental changes (Storey 1958).

4.6.5.2 Derivation of Floral Types

The evolution and derivation of the pistillate (Type I) and staminate (Type V) flowers started basically from a common ancestor, namely, the elongata hermaphrodite

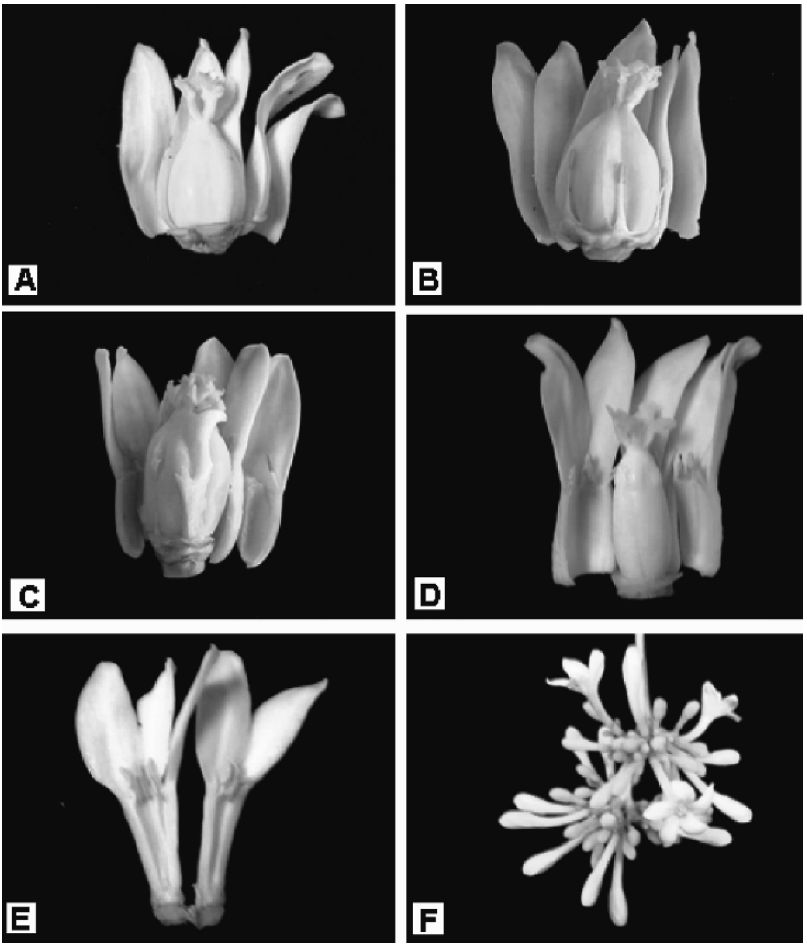

Fig. 4.1 Flower types of Carica papaya (**A**) Type I Pistillate; (**B**) Type II Pentandria ; (**C**) Type III Carpelloid; (**D**) Type IV Elongata ; (**E**) Type IV+ Barren elongate and (**F**) Type V Staminate (*See* Color Insert)

flower (Type IV) (Storey 1969b). From the elongata flower, two phylogenetic lines diverged intra-specifically, each terminating with the derivation of a unisexual form.

The staminate flower was derived along classical lines, namely, with the phylogenetic loss of the gynoecium without appreciable disturbance to other floral organs. Transitional forms of hermaphrodite flowers leading to complete maleness showed reduction in ovary size, numbers of stigmatic rays, dorsal vascular bundles, carpels and placenta (Nakasone and Lamoureux 1982).

Derivation of the pistillate flower, on the other hand, represents a departure from the classical theory. It arose not through the loss of stamens from the hermaphrodite

(elongata) type but by the incorporation or fusion of the stamens to the ovary tissues. The sequence leading to the derivation of the pistillate flower began in the upper whorl of five stamens of the elongata flower with the stamens fusing with the ovary, thus, leading to the formation of the intermediate pentandria (Type II) flower. This is the start of the final steps to the formation of the pistillate flower. After further fusion of the remaining five stamens to the ovary is complete, the pistillate flower is the result. The process of fusion of the stamens to the ovary is called carpellody of stamens. Between the elongata flower and the pistillate flower, many intermediate, carpelloid forms therefore exist, depending on the number of stamens that are fused. These flowers develop into misshapen or 'cat-faced' fruits that are not marketable.

With regard to the female or pistillate flower, its morphological structure is strongly fixed genetically in the female tree. Therefore, unlike hermaphrodite trees where sex reversal is commonplace, the pistillate tree is virtually unknown to undergo any sex change (Storey 1969b).

4.6.5.3 Environmental Influences on Sex Expression

Although the basic sex types in papaya are genetically determined, certain male and hermaphrodite trees have been known to undergo sex reversal under the influence of environmental changes (Storey 1958).

Temperature: Cool temperatures like those experienced during the winter months in the subtropics promote femaleness in hermaphrodite trees. The flowers revert from the Type IV hermaphrodite that has 10 stamens to a Type III or carpelloid form with 6–9 stamens or the Type II (pentandria) having 5 stamens. This reduction in the number of stamens is brought about by the fusion of stamens to the ovary wall. At high elevations, namely, cooler temperature, Solo papayas have a greater number of Type II and Type III fruits that are not marketable (Awada 1958). At the other extreme, warm temperatures tend to promote the production of Type IV+ (barren) hermaphrodite flowers resulting in sterility of the trees. When these conditions persist over a period of time, a production gap (sterility skip) is evident along the trunk. Allan et al. (1987) reported that male trees also showed reversion to femaleness under cool temperatures. Night temperatures of 12 °C and short daylengths caused reversion of sterile staminate (Type V) flowers to fertile, elongate type hermaphrodite (Type IV) flowers.

Moisture and Nitrogen: High soil moisture and nitrogen levels that promote vigorous plant growth encourage the expression of femaleness. Hermaphrodite trees stressed by drought produced more sterile Type IV+ flowers, while consistently high moisture levels promoted the production of hermaphrodite flowers with reduced number of stamens (Type II and Type III) (Awada 1961). Increased nitrogen application from 0.1 to 2 lb/tree at every 6-week interval brought about a 58% increase in fruit rejects arising from carpelloid fruits (Awada et al. 1979). Moisture and nitrogen levels affect the well-being and vigour of trees, indirectly bringing about reversal in sex. Vigorous growing trees during the first year of bearing had higher incidence of carpellody than subsequent harvests (Chan 1984).

4.7 Genetics of Important Characters

The chromosome number of the papaya is $2n = 18$. There are no known polyploid varieties.

4.7.1 Flower Types and Sex Expression

Sex of papaya is determined by monogenic inheritance involving three alleles (Hofmeyr 1938). The alleles are M for male, M^H for hermaphrodite and m for female. All homozygous dominants, namely, MM, MM^H and M^HM^H, are lethal to the zygotes. Therefore male genotypes (Mm) and hermaphrodite (M^Hm) are enforced heterozygotes while the female genotype (mm) is a double recessive.

Table 4.1 shows the sex segregation obtained from eight possible combinations. For gynodioecious varieties such as 'Sunrise Solo' and 'Eksotika', it is desirable to have a high proportion of hermaphrodite trees in the orchard because the pyriform hermaphrodite fruits fetch higher prices. In this case, selfing hermaphrodite flowers or hybridisation of hermaphrodite flowers with hermaphrodite pollen should be used in the production of seed. Seed derived from these cross combinations will have twice the number of hermaphrodites compared with females.

For dioecious varieties, the preferred combination for seed production is *mm* x *Mm*, namely, to use pollen from male flowers for crossing female flowers. A ratio of 1:1 male to female will be obtained. This high proportion of males in the seed far exceeds the amount that is required for pollination. Normally for dioecious varieties, a higher density is planted in the field with subsequent culling of male trees to about 10% when the sexes can be identified (Agnew 1968).

It is possible to self or cross males (combinations three and four) only if there is reversal of sex from a staminate flower to a form that has a functional ovary. This reverted 'sexually ambivalent male' (SAM) has been used for development of inbred lines in breeding of dioecious papaya (Aquilizan 1987). Night temperatures of 12 °C and short daylengths can also bring about the 'SAM' condition (Allan et al. 1987).

Table 4.1 Pollination combinations and sex segregation in papaya

	Segregation ratios			
Pollination combination	Female (mm)	Herma (M^Hm)	Male (Mm)	Non-viable zygotes (MM, M^HM, M^HM^H)
1. $mm \times Mm$	1		1	
2. $mm \times M^Hm$	1	1		
3. Mm (selfed)	1		2	1
4. $Mm \times Mm$	1		2	1
5. M^Hm (selfed)	1	2		1
6. $M^Hm \times M^Hm$	1	2		1
7. $M^Hm \times Mm$	1	1	1	1
8. $Mm \times M^Hm$	1	1	1	1

Crosses between females and hermaphrodites may be used for hybrid seed production because it obviates emasculation when female flowers are used as the maternal parent. The disadvantage, of course, is the high proportion of females in the seed. With regard to other crosses, namely, males with hermaphrodite flowers, these produce variable, trioecious populations but are not commonly used either for commercial seed production or in breeding.

There are no reliable practical methods for determining sex at the seedling stage. Sex is determined at flowering time, usually 4–6 months after planting. Multi-point planting of three or more seedlings per hill followed by culling, once sex is determined, is a common practice to achieve high hermaphrodite stands in orchards. Molecular tools (i.e. micro-satellites and PCR-based techniques) can be used to predict sex in papaya at the seedling stage but these cannot be economically applied on a commercial scale.

4.7.2 Fruit Size and Shape

There is a wide variation in fruit size in papaya, ranging from about 5 cm in diameter and 50 g weight to over 50 cm in length and 10 kg or more in weight. Size preferences vary among countries and markets. Export markets in USA, Europe and China prefer the small fruits of the 'Solo' and 'Eksotika' types while the domestic market in Malaysia prefers the medium fruit of the 'Sekaki' variety. Overly large fruits have lost out in popularity because of the inconvenience in handling and generally poorer eating quality. Fruit weight is a quantitative character determined genetically by multiple alleles. The weights of hybrid fruit commonly lie at or near the geometric mean of the parents. However, in certain crosses, heterosis in fruit weight over the better parent had been obtained (Chan 2001), indicating the presence of over-dominant gene action.

Fruit shape in papaya is a sex-linked character. The female flower has a globose ovary that develops into round or ovoid fruit. In contrast, the elongata or hermaphrodite flower has a slender, tapering ovary and this subsequently develops into a fruit that is elongated and cylindrical or pyriform in shape, depending on the variety of papaya.

Fruit shape preference varies among countries. In Australia, for example, the round female fruit from dioecious varieties are preferred, while for the majority of other papaya-growing countries like Hawaii, Brazil, India and the Southeast Asian countries including Malaysia, the gynodioecious varieties bearing elongated hermaphrodite fruit are more popular. In Malaysia, the hermaphrodite, pyriform fruit of the 'Eksotika' variety fetches a premium price that is more than twice that of the round female fruit, although there is no difference in the eating quality and total soluble solids content of fruit between the two sexes (Chan 1986).

4.7.3 Precocity and Plant Stature

Precocity or earliness of bearing and height at initial fruiting are important factors in papaya production. Precocity or earliness to fruiting is a factor of the number

of nodes produced before the first flowering node, while the number of nodes to flowering and the inter-node length determine height to first fruit (Nakasone and Storey 1955; Lim and Siti Hawa 2005). These characters are governed by additive gene effects with the hybrid having the geometric mean between the two parents (Nakasone and Storey 1955). Subhadrabandhu and Nontaswatsri (1997) reported that the height of the first flower and number of nodes to fruiting are controlled both by additive and non-additive genes. All the hybrids produced from the tall bearing parent 'Eksotika' have mean values that are skewed more towards that parent. Lim and Siti Hawa (2005) studied the earliness in flowering and dwarfism of accessions in the MARDI germplasm and concluded that inter-node length have strong correlation with plant height and the number of nodes to first flower was correlated to earliness in flowering (precocity).

4.7.4 Carpellody of Stamens

Carpellody of stamens is the development of misshapen or 'cat-faced' fruits due to fusion of the stamens to the ovary tissues in hermaphrodite flowers. High heritability ($h^2 = 82\%$) was obtained for carpellody, but effective phenotypic selection may be interfered by the 'change-in-rate' type of interaction between genotype and plant age (Chan 1984). Selection against this trait must be done early to coincide with the period of rapid growth and development when expression of carpellody is highest. Increased expression of carpellody may also be brought about by rapid growth due to cool temperature (Awada 1958), high soil moisture (Awada 1961) and high nitrogen application (Awada and Ikeda 1957).

4.7.5 Flavour and Total Soluble Solids

Flavour and total soluble solids are important traits that influence the eating quality of papaya. Flavour and odour can range from pleasantly aromatic as in the 'Solo' and 'Eksotika' varieties to undesirably musky as in the 'Maradol' variety. Muskiness has been reported to be due to the homozygous recessive allele of a single gene that could easily be bred out of a line (Storey 1969a). Total soluble solids content is usually associated with sweetness and may range from 8°Brix or less in nondescript cultivars to about 16°Brix or more in the 'Solo' and 'Eksotika' varieties. This trait is governed by quantitative genes with additive effects and hybrids are expected to have intermediate values between the parents (Subhadrabandhu and Nontaswatsri 1997).

4.7.6 Flesh Colour

Papaya flesh colour ranges from pale yellow to deep red with varying intermediate shades. In Malaysia, the preference is definitely for red-fleshed varieties. Flesh

colour is governed by a single gene with yellow (*R*) being dominant over red (*r*). Varying intermediate shades of pink may be attributed to modifier gene effects. All red-fleshed (*rr*) varieties like 'Sunrise Solo' and 'Eksotika' will breed true for flesh colour but progenies of heterozygous (*Rr*) yellow F_1 hybrids like 'Rainbow' will segregate in this trait.

4.7.7 Fruit Skin Colour

The skin colour of papaya fruit is usually green when immature, changing to yellow or reddish-orange when fully ripened. The 'Morib' is an interesting local selection that has attractive yellow fruit skin even at the immature stage. The tree is dwarfed and has yellowish green foliage. Fruit skin colour is governed by a single gene with green (*G*) being dominant and yellow expressed in a double recessive (*gg*). This attractive trait has been transferred to the 'Sunrise Solo' after hybridisation with the Morib, followed by selection for promising yellow-skinned progenies in subsequent inbred generations. The F_1 between 'Sunrise Solo' and Morib was an all green fruit population, while the F_2 generated a typical 3:1 segregation of green: yellow progenies. An inbred line called 'Niensee' that has most of the 'Solo' characteristics but with yellow fruit skin was subsequently selected (Fig. 4.2). The constraint in commercialisation of this attractive variety lies in the difficulty in identifying the appropriate maturity index of the fruit. The 'colour breaker' stage normally used for fruit harvesting is quite impossible to determine against an all yellow background.

4.7.8 Disease Resistance

4.7.8.1 Papaya Ringspot Virus Disease

The papaya ringspot virus (PRSV) is the single most devastating disease of papaya in the world. The virus belongs to the potyvirus group, which is readily transmitted by aphid vectors in a non-persistent manner. Control is made even more difficult because of the existence of alternate hosts in Cucurbitaceae. The most prominent tell-tale symptom is the dark green concentric rings on the fruit, hence the name for the disease. Infected trees are necrotic and eventually die and fruit from diseased trees are low in sugar. Some tolerant varieties have been reported such as 'Cariflora' (Conover et al. 1986), 'Tainung 5' (Lin et al. 1989) and 'Sinta' (Villegas et al. 1996). Chan (2004a) developed four lines with good tolerance to PRSV from a single seed descent programme involving the 'Eksotika' and the resistance donor 'Tainung 5'. Drew et al. (2005) successfully obtained inter-generic hybrids between *C. papaya* and PRSV resistant wild relative *V. quercifolia* and then back-crossed to *C. papaya* to get the BC_1 population. There was variability in development of PRSV symptoms in the BC_1, but one fertile resistant plant was selected that holds promise for development of a commercial variety. In genetic modification, 'Rainbow' is the

Fig. 4.2 'Niensee', an interesting genotype with yellow skinned fruit (*See* Color Insert)

world's first commercial transgenic papaya developed with coat-protein mediated virus resistance by the University of Hawaii (Gonsalves 1998). 'Rainbow' is not cultivated anywhere else outside Hawaii because of several reasons: respect for intellectual property, government policies preventing introduction and testing of GM varieties and most importantly the resistance of 'Rainbow' has been shown to be specific to the virus strains in Hawaii only. A number of countries like those in the Papaya Biotechnology Network of Southeast Asia have started their own programmes to develop papaya varieties with PRSV resistance. All are transforming with their own local strain of virus because the resistance of transgenic papayas like 'Rainbow' seemed to indicate strain specificity (Tennant et al. 1994). The danger in using the coat protein of only one specific strain like the PRSV-YK in Taiwan for transformation is the breakdown of resistance by other PRSV strains or new unrelated viruses like the papaya leaf distortion mosaic virus (Chen et al. 2002).

Estimation of heritability of tolerance to PRSV by parent/offspring regression conducted on 35 papaya lines (Chang and Guo 2002) indicated $h^2 = 61\%$ on disease index. Yield and total soluble solids were positively correlated to the degree of tolerance to PRSV.

4.7.8.2 Bacterial Dieback

A 'bacterial decline' of papaya caused by *Erwinia* sp appeared in the West Indies at the end of the 1960s and later spread to the Virgin Islands and Venezuela (Ollitrault et al. 2005). A similar bacterial dieback disease caused by *Pantoea agglomerans* was reported in Malaysia in 2003 (Mohamed 2005). Typical early symptom is collapse of the petiole near the lamina that leads to complete rotting and disintegration of the crown. Circular dark spots appear on the fruit that develop later into deep black lesions or holes. Tolerant dioecious accessions have been found in Venezuelan and Guadeloupean germplasm while all commercial cultivars are highly susceptible. The level of tolerance is variable and inherited in an additive manner subject to strain virulence, but genotypic ranking for tolerance remains the same. Tolerance is transmitted in a co-dominant way involving a few genes, with F_1 hybrids intermediate in reaction between susceptible and tolerant parents (Ollitrault et al 2005).

4.7.8.3 Fruit Freckle

Fruit freckle is widespread in the 'Solo' papayas including 'Eksotika' and is considered a disease of unknown cause in Hawaii (Hine et al. 1965). The symptoms appear as superficial water-soaked spots of variable sizes, more apparent on the exposed surface of the maturing fruit on the tree and apparently associated with the lenticels of the fruit skin. The freckles do not affect the eating quality of the fruit, but the cosmetic appeal of the infected fruit may be considerably reduced. Freckle is genetically controlled, as shown by Chan and Toh (1988), when three back-crossed sib lines – Line 7, Line 19 and Line 20 – showed distinct differences in response to this disorder over two seasons. Wet season caused higher severity, not an increase in the number of freckle spots, but in the enlargement of freckle size. Line 20 was the most susceptible, Line 7 intermediate while Line 19 was the most resistant. The progeny of Line 19× Line 20 showed very good tolerance, shifting more towards the resistant parent than the mid-value of both parents, suggesting partial dominance of the resistant genes (Chan and Toh 1998).

4.8 Varieties

Storey (1969a) reported that the only bona fide varieties of papaya in existence were 'Solo' and 'Bush' of Hawaii and 'Hortus Gold' of South Africa. Since then, however, numerous distinct, true-breeding varieties have been developed from many parts of the world. Many are from systematic breeding programmes, while others are from judicious selection efforts by growers. Recently, biotechnology tools have been used to develop transgenic varieties. Papaya varieties can be self-pollinated (in which case they are purelines) or cross-pollinated. In general, gynodioecious varieties (having hermaphrodite and female trees) are self or cross-pollinated, while the dioecious varieties (having male and female trees) are enforced cross-pollinators.

4.8.1 Gynodioecious Varieties

4.8.1.1 'Solo' Types

Gynodioecious varieties are also known as 'bisexual' or 'hermaphrodite' where no male trees are present. The most popular hermaphrodite varieties in the world today are those from the 'Solo' family, so called because of the small fruit size suited for one serving. Within the 'Solo' are many lines such as 'Line 5', 'Line 8', 'Line 10', 'Kapoho', 'Waimanalo' and 'Sunrise' (Yee et al. 1974), 'Wilder' and 'Higgins' (Nakasone et al. 1974), 'Sunset' and 'Kamiya' (Fitch et al. 2002). 'Solo' is typically small-fruited around 500 g, pyriform shaped with a distinct 'neck' for hermaphrodite fruits and usually with yellow to yellow-orange flesh (except for Sunrise and Sunset that are orange-red). The flavour is distinct and pleasant with usually high TSS of 13–16%. Lines 5, 8 and 10 were the earliest recommendations by the University of Hawaii and these have been phased out or restricted to domestic markets because the fruit softens quickly and has poor keeping quality. Wilder and Higgins too did not make commercial impact because of high occurrence of carpellody. Trials in Malaysia recorded occurrence of 40% and 75% respectively for these two varieties, although Nakasone et al. (1974) have indicated less than 10% when grown in Hawaii. Below are descriptions of the 'Solo' types of commercial importance.

Waimanalo: *(Waimanalo Solo X-77)*. Waimanalo was selected from crosses between 'Betty' from Florida and 'Line 5' and 'Line 8' Solo strains (Yee et al. 1974) and released by the Hawaii Agricultural Experiment Station in 1968, superseding the early 'Solo' lines. It bears fruit much lower to the ground than other Solo types. The fruit is medium-sized, weighs 600–700 g, roundish with a short neck with smooth, shiny 'freckle-free' skin. The flesh is orange-yellow, thick and fairly firm with TSS around 14–17%. It is very tolerant to *Phytophthora* root rot as well as yield decline due to repeated cropping in the same area (Nakasone and Aragaki 1973).

Sunrise and Sunset Solo: These two are the only 'Solo' types that have red flesh. 'Sunrise' (formerly 'HAES 63-22') is the most popular of the 'Solo' and is grown worldwide because of its wide adaptability. The fruit is small, weighs around 400–500 g, elongate with a distinct neck with smooth and usually 'freckled' skin. The flesh is orange-red, fairly soft with an exquisite pleasant fragrance and high TSS around 15–17%. Sunrise was used as a recurrent parent in a back-cross breeding programme to improve Malaysian cultivars (Chan 1987). 'Sunset' is of the same parentage as 'Sunrise' and is similar in all respects except for smaller fruit size of about 350–450 g. 'Sunset' was the target variety used in transformation for cp-mediated virus resistance resulting in selection of the first papaya transgenic 'SunUp' (Gonsalves 1998).

Kapoho: This is the major cultivar grown in Hawaii before the industry was crippled by PRSV in the mid 1990s. Many hectares are now superseded by the resistant genetically modified 'Rainbow' especially in the hotspot areas (Mochida 2005). However, 'Kapoho' continues to be the dominant variety grown in Hawaii for export especially to Japan that does not accept GM papayas. The 'Kapoho'

fruit is small, weighs 400–550 g and pyriform. The flesh is orange-yellow, thick and very firm with TSS around 14–16% and strong flavour. It has very good shelf life and withstands well during shipping. However, it is sensitive to drought that can drastically reduce fruit to unmarketable sizes. In Hawaii, it is grown primarily in the eastern part of the island where annual rainfall is more than 2,500 mm (Yee et al. 1974). 'Kapoho' was used to cross with the transgenic 'SunUp' to produce the commercial PRSV resistant 'Rainbow'.

Kamiya: 'Kamiya' is a selection from Waimanalo. The tree is a dwarf and high yielding. It is a large-fruited variety with distinct, blocky shape and a very short neck. Its flesh is thick, deep orange-yellow, firm and juicy and has a flavour reminiscent of coconut or mango (Fitch et al. 2002).

Eksotika, *Eksotika II*: 'Eksotika' and 'Eksotika II' are the flagship varieties for export in Malaysia (Chan 2004b; Fig. 4.3). 'Eksotika' has similar features as 'Sunrise Solo' because 'Sunrise' was used as a recurrent parent in the backcross programme that developed this variety (Chan 1987). 'Eksotika' is a good bearer, yielding about 50–60 tons/ha/year. The fruit is medium size (600–800 g) with orange-red flesh (Fig. 4.4). It has the Solo pleasant aroma and high sugar content of 12–14° Brix but does not keep well because of its soft texture. It is

Fig. 4.3 'Eksotika', a small-fruited Solo type

Fig. 4.4 Small fruited 'Solo' type papaya (*See* Color Insert)

also quite susceptible to fruit freckles and malformed top disease (Chan and Mak 1993a). 'Eksotika II' is a F_1 hybrid between 'Line 19' and 'Line 20' (Chan 1993). Compared with its predecessor, 'Eksotika II' has higher yield due to slightly larger fruit (600–1,000 g), firmer flesh for longer storage and less prone to fruit 'freckles'.

4.8.1.2 Large-Fruited Types

There are several gynodioecious papaya varieties that are large-fruited and bear little resemblance to the 'Solo' types. These are usually grown and consumed locally because the large fruit is inconvenient to handle and pack for export. The varieties in the ASEAN region are 'Batu Arang', 'Subang 6' and 'Sitiawan' from Malaysia, 'Kaegdum'('Khaek Dam'), 'Kaegnuan', 'Koko' and 'Sainampeung' from Thailand, 'Cavite Special' from the Philippines, 'Dampit', 'Jingga' and 'Paris' from Indonesia (Chan et al. 1994). The other large-fruited varieties popular in other parts of the world are 'Coorg Honey Dew', 'Maradol', 'Red Lady #786' and the 'Tainung' series 1–3. They generally have elongate or cylindrical fruit, ranging from 1 kg to 6 kg in the case of 'Cavite Special'. 'Khaek Dam' is Thailand's best known variety. It is vigorous, bears red-fleshed fruit of about 1.2 kg with 10.6% total soluble solids content (Subhadrabandhu and Nontaswatsri 1997). 'Coorg Honey Dew' is a popular selection from 'Honey Dew' made by the Indian Institute of Horticultural Research. The fruit is long, weighs 2–3.5 kg with yellow flesh and a large cavity and keeps fairly well (Morton 1987). 'Maradol' originates from Cuba, but is now grown widely in Mexico and South America. It is short-statured and bears fruit very close to the ground. Fruit weighs 1.5 kg, attractive with firm red flesh with 10–11% TSS content. It has a characteristic strong flavour described by some as 'musky' and the fruit is quite susceptible to anthracnose when grown in the humid tropics. 'Red Lady #786' is a red fleshed fruit averaging 1.5–2 kg and quite tolerant to papaya ringspot and ripe-rot diseases such as anthracnose. 'Tainung' series 1–3 are popular varieties

in Taiwan and to some extent in the Caribbean, South America and South Africa. ‘Tainung 1’ and ‘2’ hermaphrodite fruits are elongate with a pointed end, weigh around 1.1 kg and red fleshed. These two varieties were introduced into South Africa in the early 1990s and because of their superior quality and consistency of fruit, have replaced most of the dioecious varieties there (Louw 2004). ‘Tainung 3’ fruit is more pear shaped, larger (1.3 kg) and orange fleshed. ‘Red Lady’ and ‘Tainung’ series 1–3 are F_1 hybrids developed by the Known You seed company in Taiwan and listed in several international seed catalogues.

Recently, Philippines introduced a new hybrid ‘Sinta’ with moderate tolerance to PRSV (Villegas et al. 1996). ‘Sinta’ is a F_1 hybrid between ‘Py-5’ and ‘Py-3’, bears fruit of 1.2–1.3 kg with yellow-orange firm flesh and with 11.9°Brix TSS. Its yield is 35–60 kg/tree in one fruiting cycle. In PRSV hotspots, ‘Sinta’ is recommended as an annual crop. Otherwise it can keep fruiting for at least three years. In Malaysia, the recent introduction is ‘Sekaki’, the ‘foot-long’ papaya in Malay, and it has replaced to a large extent the older varieties like ‘Subang 6’. ‘Sekaki’ (Fig. 4.5), sometimes called the ‘Hong Kong’ papaya, is a prolific bearer (60 tons/ha/year) with medium sized cylindrical fruit of 1.5–2 kg. The tree is short statured, bears low to the ground and is resistant to malformed top disease. ‘Sekaki’ fruit is attractive with a smooth, even-colour and freckle-free skin. The flesh is red, firm but sugar content is not high at 10° Brix or less (Chan 2001). ‘Sekaki’ has a small export market, mainly to Singapore and Hong Kong, but most of the fruit are sold locally.

In general, the large-fruited gynodioecious varieties do not have the quality, flavour and sweetness of the ‘Solo’ types. Some, as in ‘Kaegnuan’, the immature fruit is consumed as vegetable in Thailand.

Fig. 4.5 ‘Sekaki’ a large fruited variety from Malaysia (*See* Color Insert)

4.8.1.3 Transgenic Papayas

'Rainbow' is the world's first commercial transgenic papaya developed with coat-protein mediated virus resistance by the University of Hawaii (Gonsalves 1998). It is an F_1 hybrid made from a cross between the transgenic 'SunUp' and the non-transgenic commercial cultivar 'Kapoho'. Transgenic varieties of 'Kapoho' and 'Kamiya' have also been developed by the introduction of coat-protein transgene from 'Rainbow' through conventional hybridisation and back-crossing. The 'Poamoho Gold' is a back-cross 1 hybrid between 'Kapoho' and 'Rainbow F2', while the 'Laie Gold' is a F_1 hybrid between 'Kamiya' and 'Rainbow F_2' (Fitch et al. 2002).

In the near future, many transgenic papaya varieties will be developed in Malaysia, Thailand, Indonesia, Philippines and Vietnam. These countries come under the ISAAA-coordinated Papaya Biotechnology Network of Southeast Asia formed in 1998 and have been actively working on the development of ringspot virus (PRSV) resistance and delayed fruit ripening (Hautea et al. 1999). Working in close collaboration with Monsanto and University of Hawaii in the PRSV resistance project, and with Syngenta (Zeneca) and University of Nottingham in the delayed fruit ripening project, several countries in the network notably Thailand, Vietnam and Malaysia, already have transgenic lines in field trials.

4.8.2 Dioecious Varieties

Dioecious varieties with male and female flowers on separate trees are enforced cross-pollinators. Dioecious commercial varieties are not common and restricted only to some countries like Australia and South Africa. The disadvantage of dioecious varieties is that 50% of the population is unproductive male trees. In commercial cultivation of dioecious varieties, a high initial stand of seedlings is first established with subsequent culling of male trees to 5–10% for pollination purpose. The advantage of dioecious varieties is that fruit size, shape and appearance is more uniform and stable because female flowers do not undergo sex reversal as in hermaphrodites. 'Hortus Gold', 'Honey Gold', 'Sunnybank', 'Hybrid No. 5', 'Cariflora', 'Co1' and 'Co2' are the known commercial dioecious varieties.

'Hortus Gold' is a South African cultivar released in the early 1950s. It is early maturing with round-oval, golden-yellow fruit weighing 0.9–1.36 kg (Morton 1987). 'Honey Gold' is a selection made from 'Hortus Gold' and has many similar features but with improved sugar content and disease resistance (Morton 1987). It is a late season bearer and its fruit fetch better prices at the tail end fruiting of other varieties. Commercial orchards of these two varieties are propagated by cuttings (Allan 1993). 'Sunnybank' and 'Hybrid No. 5' were popular cultivars in Queensland, Australia (Agnew 1968). 'Sunnybank' strain S7 was selected at the Redlands Horticultural Research Station, while 'Hybrid No. 5' is a F_1 hybrid between 'Bettina 100A' and 'Peterson 170'. These Queensland varieties bear round to ovoid fruit, about 1–2 kg in weight with attractive clear smooth skin that colours uniformly on full ripening.

The flesh is yellow and usually soft with 11–12% TSS. 'Cariflora' is fairly dwarf and bears small rounded fruit (0.5–0.8 kg) with a slightly pointed end. The fruit has yellow flesh, soft texture and low TSS (9.5–10.8%). It is tolerant to papaya ringspot virus and is recommended for cultivation in diseased areas in Florida and the Caribbean (Conover et al. 1986). It was also used as a parent for improving PRSV tolerance of the 'Eksotika' papaya in Malaysia (Chan 2004a). 'Coimbatore 1 and 2' or 'Co1' and 'Co2' were developed at the Tamil Nadu Agricultural University. The dwarf trees bear fruits low to the ground. Fruits are medium sized, 1.5–2.5 kg with yellow, sweet flesh. (Morton 1987). 'Co2' is also recommended for papain extraction.

4.9 Propagation

4.9.1 Seed

Papaya is almost entirely propagated from seed in commercial cultivation. Sound seed usually germinate after two weeks in polybags and are ready for transplanting at the 8–12 leaf stage after another 6 weeks. The cost of production for each seedling is estimated to be 35 sen (about USD 0.1) (Chan et al. 1991) and this works out to about 3.5% of the total production costs for papaya (estimated at USD 2.8/tree over 2 years).

The seeding rate for papaya is very low. This is because dry papaya seeds are relatively light, weighing about 14.5 g per 1,000 seeds. Furthermore, the density of the crop at 2,000 trees/ha is relatively low compared with cereals and horticultural crops like vegetables. For establishing a hectare of papaya, about 3,000 seeds or only 50 g are required (Chan 1994). This could be increased to 75–100 g if multiple planting per point is practised.

4.9.2 Vegetative Propagation

Vegetative propagation is the norm in perpetuation of perennial fruit trees and many horticultural crops, particularly ornamentals. The main advantage in propagation by vegetative means is that it allows fixing of the maternal genotype and faithfully reproducing it from one generation to another. Several methods of vegetative propagation are available for papaya. Although these are still not widely used in commercial plantings, there are good prospects and potential for their adoption and utilisation in the near future.

Allan (1964) was the first to report success in propagating papaya by cuttings. Large, leafy, lateral shoots that developed after winter were initially used as cuttings for rooting under intermittent mist. In subtropical countries, the cool winter checks growth and temporarily overcomes apical dominance, resulting in the proliferation of lateral shoots. Availability of cuttings became less season-dependent when they

were induced from vigorous one- to two-year-old trees by topping off the shoot terminus to remove apical dominance. The method for induction and proliferation of suitably sized lateral shoots for cuttings was improved further with the application of cytokinin and gibberellic acid mixtures (Allan 1993). The ideal size of cuttings would be 50–150 mm long and 8–12 mm diameter with 4–5 leaves. These are harvested, trimmed to leave 3–4 small leaves and treated with fungicide and a basal dip in Indolebutyric acid (IBA) to encourage rooting before they are planted in intermittent mist beds with bottom temperature of 30 °C. The cuttings will root in about three weeks.

In field performance, Fitch et al. (2002) reported that clonally propagated 'Rainbow' transgenic papaya flowered 1–3 months earlier and 30 cm lower compared with seedlings. Clonal plants yielded significantly higher than seedlings and the difference was more marked in less favourable environments.

Papaya can also be propagated by grafting. Airi et al. (1986) successfully cleft-grafted scion shoots from cultivars Co-1 and Honey Dew onto uniformly established seedlings. Chong et al. (2005) also used cleft grafting to establish clonal hermaphrodite 'Eksotika' plants and reported an initial success of 80%, although this was reduced later due to infection of soft-rot fungi. Patch and T-budding can also be used, but the success rate was poorer than with cleft grafting. In Malaysia, some papaya growers have used field grafting to replace female trees of the 'Eksotika' cultivar in the orchard (Cheah et al. 1993). As soon as the sex of the trees can be determined, the female trees are side-cleft grafted with scion shoots (basal diameter 2–3 cm) harvested from hermaphrodite 'Eksotika' trees. When the union is established in about 2–3 weeks, the female tree is cut back to about 60 cm from the ground to allow the scion to take over. The time in bearing and yield of these in-field grafted hermaphrodites are not significantly different from seed-propagated trees. This practice is economically justifiable because of the much better price paid for hermaphrodite 'Eksotika' fruit.

4.9.3 In Vitro Propagation

Early successes of in vitro propagation of papaya were reported by Mehdi and Hogan (1976) and Yie and Liaw (1977). However, seedling tissues were used as primary explants and, as such, the application is limited because the sex of the plants, which is of prime importance, could not be ascertained. Later, Litz and Conover (1978) reported successful regeneration of papaya plantlets by culturing apices of mature, field-grown papaya plants in modified Murashige and Skoog media. This success stimulated more in vitro research on papaya because of the prospects for mass propagation, predictable plant sex and greater uniformity of the crop.

In Malaysia, in vitro propagation research was carried out using primarily the 'Eksotika' papaya (Nathan and Tan 1989; Chan and Teo 2002). Field trials of in vitro plantlets indicated that they propagated true to sex without somaclonal reversion. Therefore, the problems of sex segregation and variation of fruit shape that

seed propagation face do not arise in micro-propagated plants. Besides greater uniformity, the other benefits are earlier and lower bearing height and improved yield (Drew 1988; Nathan and Tan 1989; Chan and Teo 2002).

Despite all the clear advantages, the use of in vitro propagated papaya in commercial plantings appears to be an exception rather than the rule. The most likely reason may be related to economics. Demand for such planting materials may not be high enough to justify the economies of scale for the large capital investment. Under limited demand, some tissue culture laboratories in Malaysia have been known to sell in vitro propagated plantlets for US 40–50 cents each. This is about five to six times the price of raising a plant from seed. Survival of micro-propagated plants ex vitro, reported to be as low as 30% (Fitch et al. 2002), may also pose a problem.

Besides the prospects of using in vitro as a method for rapid mass propagation of papaya, it has also been used in embryo rescue to obtain plants from otherwise incompatible inter-generic crosses between *C. papaya* and other *Carica* (now *Vasconcellea*) species (Manshardt and Wenslaff 1989b), rapid disease resistance screening (Sharma and Skidmore 1988), anther culture for generating haploid papaya lines (Tsay and Su 1985) and transformation and regeneration of genetically modified papaya for transferring resistance to papaya ringspot virus disease (Pang and Sanford 1988).

4.10 Breeding and Genetics Research

4.10.1 Germplasm

Germplasm is the genetic resource or genebank upon which breeders will draw parents for hybridisation and improvement of varieties.

Several countries have established papaya germplasm collections to support varietal improvement programmes. In Malaysia, MARDI currently has 52 accessions of papaya, 19 of which are local while the rest are introduced mainly from Hawaii, Brazil, Australia and Southeast Asia. Chan (1985a) had characterised the germplasm for fruit quality traits such as total soluble solids and fruit weight, and also earliness and height of fruiting and reported wide genetic variation that is useful for improvement of local varieties. Lim and Siti Hawa (2005) studied the earliness in flowering and dwarfism of accessions in the MARDI germplasm and concluded that inter-node length have strong correlation with plant height and the number of nodes to first flower was correlated to earliness in flowering (precocity). The most dwarf accessions were ‘Baixinho Sta Amalina’ and ‘Puerto Rico 217’ and these may be useful as parents for improving precocity and dwarfism of local cultivars. Suhana and Mohamad Bahagia (2005) studied variation of fruit characters in the same germplasm and reported wide variation in fruit size parameters and total soluble solids (TSS). There was strong negative correlation between fruit size parameters and TSS, implying the difficulty in selection of genotypes with sweet, large fruits.

The USDA/ARS National Germplasm Repository at Hilo, Hawaii, currently holds 37 accessions of *C. papaya* and 13 species of *Vasconcellea* (Zee, F., *pers*

comm. 2006) which was a drastic reduction from 153 accessions reported 2 years earlier (OECD 2004). The main reason for the decline was the PRSV disease. Other large holdings of accessions are those in India (90 *C. papaya*), Brazil at EMBRAPA mandioca e fruticola (96 accessions), Bahia State at EBDA (82 accessions), Campinas, SP at IAC (169 accessions) and Columbia at UN Mendellin and CORPOICA (83 accessions and additional accessions at other locations). The FAO's Seed and Plant Genetic Resources Service (AGPS) has information on the locations of papaya germplasm in the world and nearly 90 research stations or seed production sites are on record (FAO 2001).

4.10.2 Conventional Breeding

4.10.2.1 Mass Selection

In many developing countries, papaya farmers were, to a large extent, responsible for improving varieties. They used simple mass selection by picking the best trees for seed propagation into the next crop cycle. Farmers at each geographical location in the country may have their own selection preferences; hence, varieties from different areas may have unique similarities that would distinguish one from another.

Mass selection can be used as a short-term programme for 'cleaning' and stabilising local varieties selected by farmers. In Malaysia, for example, 'Subang 6', selected for fresh fruit, and 'Sitiawan', for processing, were recommended as interim varieties to 'kick-start' the industry (Chan and Ooi 1975).

4.10.2.2 Back-Cross Breeding

Back-cross breeding is usually used for transferring a single character from the non-recurrent donor parent to the recurrent parent. It was used successfully for increasing the fruit size and adaptability of 'Sunrise Solo' in Malaysia (Chan 1987). This programme used 'Subang 6', which has local adaptability and large fruit, as the non-recurrent parent and the 'Sunrise Solo', which has excellent eating qualities, as the recurrent parent. The variety developed from this programme was called the 'MARDI Backcross Solo', which was later named 'Eksotika' when it was released in 1987.

Back-cross breeding was also used in development of 'Poamoho Gold' that is a back-cross 1 hybrid between 'Kapoho' and 'Rainbow F2' (Fitch et al. 2002). Back-crossing was also carried out for transferring PRSV resistance from the donor parent *Vasconcellea quercifolia* to the cultivated varieties (Drew et al. 2005).

4.10.2.3 Hybridisation

Heterosis or hybrid vigour is the increase in yield, size or robustness of an organism arising from bringing together of unlike gametes to form a hybrid. Heterosis breeding is so significant in agriculture that there are hardly any commercial crops

that had not included development of hybrids in their breeding programmes at one time or another. Many cereals like corn, sorghum, millet, barley, wheat, rice and horticultural crops like vegetables, fruits, flowers as well as pasture crops and fodder grasses have benefited in increased plant robustness and yield from heterosis breeding. The added advantage in hybrid varieties is the propriety and monopoly in seed production. By maintaining strict security of the inbred parental lines used in production of the hybrid seeds, the dissemination and sale of these seeds can be regulated.

The most important commercial papaya cultivars in the world today are inbred purelines such as 'Kapoho', 'Sunrise Solo' and the 'Eksotika' that are propagated from generation to generation from seed obtained by selfing hermaphrodite flowers of gynodioecious varieties. There appears to be no inbreeding depression in papaya during selfing of gynodioecious populations (Hamilton 1954) or dioecious populations (Aquilizan 1983). There was, until very recently, little interest in F_1 hybrids of papaya but the works of Chang and Wu (1974) and Subramanyam and Iyer (1984) demonstrated significant heterosis in crosses between varieties and Mekako and Nakasone (1975) reported heterosis from inter-specific crosses. Chan (1992) reported heterosis in yield and fruit quality in F_1 hybrids developed from closely related sib crosses. Using wider crosses, the hybrids were generally superior to inbreds in characters related to vigour, precocity and yield. The mean heterosis for yield in the first year of harvest was an astounding 175% over the mid-parent value (Chan 1995). Hybrids compared with inbreds seemed to have better 'agronomic' stability in that they responded better to improvements in environments. Under harsh environments, heterosis was not significant, but the gap between inbreds and hybrids widened considerably as environments become more favourable for papaya growing (Fig. 4.6). The high yield and early cropping characteristics of the wide-cross hybrids may also be useful in escaping the PRSV disease. An annual economic harvest may be obtained before the trees are debilitated by the disease.

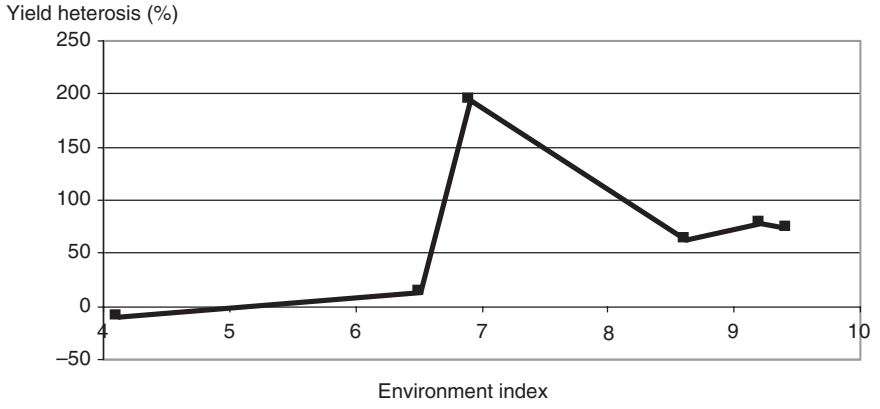

Fig. 4.6 Heterosis in relation to environment index

There are now increasingly more F_1 hybrids in the market today. 'Eksotika II' from Malaysia (Chan 1993) and the PRSV tolerant 'Sinta' from the Philippines (Villegas et al. 1996) are well-known F_1 hybrids. A number of papaya varieties in seed catalogues such as 'Tainung #1–3', 'Red Lady #786' and 'Golden Maradol' are also F_1 hybrids. 'Rainbow', the GM papaya with PRSV resistance, is a F_1 hybrid between the transgenic 'SunUp' and the non-transgenic commercial cultivar 'Kapoho' (Gonsalves 1998). The latest F_1 GM hybrid from Hawaii is the 'Laie Gold' developed from a cross between 'Kamiya' and 'Rainbow F_2' (Fitch et al. 2002).

4.10.2.4 Field Evaluation for Varietal Stability

Before the recommendation and release of a new variety, there is a need to test the stability and performance of the selected genotypes over several locations or seasons. Genotypes often do not show the same relative performance over all the environments. This lack of additivity for genotypic and environmental effects leading to inconsistency in performance of genotypes over environments is commonly termed genotype × environment interactions (GE interactions).

The occurrence of significant GE interaction in varietal trials, over a number of locations (spatial) or over a number of seasons or years (temporal), will complicate selection and recommendation of advanced breeding lines or varieties. This is because the superiority of varieties is location- or season-specific and generalization of their superiority becomes impossible or highly unreliable. Specificity presents a pressing issue and a great challenge to papaya breeders who would like to develop high-yielding varieties that perform consistently over a range of environments.

Many methods have been used in the papaya field trials to select for stable, consistent as well as high quality and high yield varieties. Stability is a word that usually appears in GE studies because the degree of stability of a genotype is linked to the magnitude of its GE interaction. A stable genotype is one that shows a very small GE interaction in the character concerned, namely, the genotype is consistent in performance regardless of the changes in a set of environments. In one of the early papaya trials, Chan and Ooi (1975) used two parameters, variance and mean of the genotype as criteria for selection. The best performers would be genotypes with low variance (high uniformity) and the most desirable mean value. 'Honeydew' was selected for good sugar content while 'Sitiawan' was selected for heavy fruit weight. CV estimates and means distribution as an analysis for stability was later proposed by Francis and Kannenberg (1978) as a simple method for selection of a large number of genotypes because the scatter diagram presents a good visual on their performance. It, however, measures the Type 1 or static stability and genotypes with this type of stability are usually poor performers and do not respond to favourable changes to the environment (Lin, Binns and Lefkovitch 1986).

Chan (1985b) analysed GE interaction and stability of several papaya varieties developed by MARDI's breeding programme using structural relationship analysis proposed by Tai (1971). Two statistics were used in the description of stability, namely, α which measures the linear response of a genotype to environment

influences and η that measures the deviation from linear response. The results showed that 'Eksotika' (then called 'Backcross Solo') had average stability for yield and total soluble solids ($\alpha = 0,\ \eta = 1$), suggesting that this variety will perform well generally over a range of environments.

The general conclusion from a series of papaya trials showed the antagonistic relationship between stability and yield (Chan 1985b). Although perfectly stable varieties may be obtained, these are frequently poor performers and high yielders are usually less stable. Chan and Mak (1996) examined several methods that may be effective for simultaneous selection for yield and stability in papaya. In the choice of methods to use for simultaneous selection of genotypes for yield and stability, the breeder will have to decide in which direction the emphasis is directed. In the case where more emphasis is given to yield performance, the mean and CV method of Francis and Kannenberg (1978) appears appropriate and convenient. In the case where stability is given emphasis, Hühn's (1979) non-parametric indices would be suitable, but would have to be used with caution because of the extreme bias for stability and total disregard for yield performance in situations where genotypes did not vary their rankings over environments. In this method too, there is some fine-tuning in emphasis for mean and stability by choosing the appropriate S_i^3 or S_i^6 indices. Finally, the rank sum and rank product proposed by Kang (1988) appears to be well-balanced and less biased in selection between mean and stability with the added advantage that the stability estimate is of the useful Type 2 or 'agronomic' stability. The rank sum index appears to give slightly more weight to selection for stability rather than yield, while in the rank product, the reverse is true.

4.10.3 Induced Mutations

Mutation breeding is of value in redressing a single weakness of an otherwise excellent variety. Induced mutation either with chemicals or irradiation causes minute changes in the genotype and with an efficient screening procedure will pick out the mutant with the improved characteristic. Irradiation induced mutation on papaya was carried out at MARDI, Malaysia. Tests on sensitivity to gamma-irradiation showed that 525 Gy and 42.5 Gy were the most suitable dosages for mass irradiation of dry and pre-soaked papaya seeds, respectively (Chan et al. 2002). In the M_1 population ('Eksotika' variety), many physiological defects were observed including stem splitting, leaf variegation, crinkled dwarfs and leaf puckering. In the M_2 population, wide variability was recorded for many traits. At seedling stage, a low irradiation of 42.5 Gy on pre-soaked seeds produced high number of M_2 progenies that were shorter and more vigorous in leaf development than those irradiated at 525 Gy and the control seedlings (Chan and Lam 2003). The distribution patterns of M_2 progenies for nine quantitative characters showed great variation with ranges often exceeding the limits of the control population. There appears to be good prospects in improving 'Eksotika' papaya, especially in the development of more dwarf trees with lower fruit bearing stature, higher total soluble solids in fruit and

larger fruit size. Several M_2 mutants also showed very good resistance to malformed top disease. However, no resistance to PRSV disease was found after inoculation and screening of 1,760 M_2 seedlings (Chan 2004c).

4.10.4 Biotechnology in Papaya Improvement

4.10.4.1 Papaya Ringspot Virus Resistance

Development of genetically modified papaya with resistance to ringspot virus in Hawaii followed the concept of parasite-derived resistance (PDR) that was successfully used for development of GM tobacco with resistance to mosaic virus. The research team consisted of molecular biologist Jerry Slightom (Upjohn), horticulturist Richard Manshardt, tissue culturist Maureen Fitch (University of Hawaii) and virologist Dennis Gonsalves (Cornell University). The coat protein (CP) gene of the mild mutant PRSV HA 5-1 was first isolated and inserted into the transformation vector pGA482GG and the resulting plasmid DNA delivered via high velocity micro-projectiles into the papaya cells. Two successful transgenic lines were obtained from the 'Sunset' varieties initially codenamed '55-1' and '63-1'. The '55-1' line from a single R_0 transformant was a female genotype. Subsequent breeding, progeny testing and selection resulted in the selection of 'SunUp', a homozygous '55-1' with double copy resistance. 'SunUp' is red-fleshed and the preference in the U.S. market is for yellow flesh. 'SunUp' was crossed with the commercial yellow-fleshed 'Kapoho' to produce the 'Rainbow', the first commercial papaya transgenic (Manshardt 1998).

Following the success of 'Rainbow', the coat-protein mediated resistance for PRSV was adopted by countries in the Papaya Biotechnology Network of Southeast Asia (Hautea et al. 1999). In Malaysia, the gene constructs from coat protein and Nib replicase genes of the local PRSV strain were made and transferred separately into tissues of 'Eksotika' papaya by *Agrobacterium* (Mat Daud et al. 2005). Thailand successfully carried out a field trial of the transgenic PRSV resistant papayas and reported that a R_2 transgenic line KN116/5 was highly resistant with only 3% infection compared with 100% of non-transformed trees (Phironrit et al. 2005). Yield was 40 times higher and fruit quality was good with TSS of 12° Brix.

4.10.4.2 Extending Storage Life

Papaya being a climacteric fruit has relatively short storage life that restricts its export to distant markets. Extending its storage life to a period that allows export by less-expensive sea transport will be effective in opening up new markets abroad. Delay in fruit ripening can be achieved through suppression of ethylene biosynthesis and reduction in the cell wall degrading enzymes. The Plant Genetic Engineering Laboratory at the University of Queensland, Australia, cloned and characterised ethylene biosynthetic genes involved in papaya ripening and produced over 100 independent transgenic lines with ACC synthase genes (Botella et al. 2005). Results

of field trials show that transgenic lines have delayed ethylene production and delayed ripening of up to 21 days. In Malaysia, Ali et al. (2000) isolated and characterised genes and gene products (mainly α- and β-galactosidase) responsible for fruit softening in papaya. They concluded that management of texture using softening genes is more complicated than delaying ripening through restricting ethylene synthesis because it involves down-regulating the activity of a number of relevant genes. Abu Bakar et al. (2000) isolated the ACC oxidase gene from cDNA libraries of ripe 'Eksotika' fruit, then cloned and characterised it and made the antisense gene cassettes for plant transformation. Transformation was done by the biolistic method using kanamycin as the selection media. Successfully transformed plants showed significant reduction in wound-induced ethylene production (Abu Bakar et al. 2001). Fifteen R_0 lines have been evaluated for phenotypic expression of delayed fruit ripening in a contained net-house trial at MARDI (Raveendranathan et al. 2002). Seven R_1 lines are now in open field (Pauziah et al. 2005). Other countries in the Papaya Biotechnology Network of Southeast Asia are in various stages of gene cloning and molecular characterisation of the ethylene biosynthetic genes and transformation of the commercial cultivar of each country. The progress in Thailand was reported by Burns et al. (2005), Philippines (Magdalita et al. 2005) and Indonesia (Damayanti et al. 2005).

4.11 Seed Production, Treatment and Storage

4.11.1 Seed Production

It is important to maintain the purity of the varieties during seed production so that the desirable qualities of the fruit are not lost. The majority of gynodioecious varieties are self-pollinated purelines and, even in cross-pollinated gynodioecious or dioecious varieties, seeds may be reproduced with good genetic purity if care is taken. The growers themselves can produce their own seed without having to buy them from nurseries. However, for the F_1 hybrid like 'Eksotika II', its seeds are difficult to reproduce because they need two inbred parents ('Line 19' and 'Line 20') to be crossed for production of the hybrid seed (Chan 1993).

4.11.1.1 Producing Seed of Self-Pollinated Varieties

The 'Solo' group consists of self-compatible gynodioecious varieties whose hermaphrodite flowers can set fruit when pollinated with their own pollen. The anthers are located very close to the stigma and as the anther sacs burst they release the pollen before the flower itself opens to ensure self-pollination.

Self-pollinated seed are reproduced with good purity by covering each hermaphrodite flower with a wax paper bag (7 cm × 10 cm) before the flower opens. This is important because if the flower had already opened, there may be a chance that pollen from other varieties may have caused fertilisation and the seeds

that develop later are not pure. The wax paper bags will also keep away excessive moisture from the flowers for good fruit set.

The paper bags can be secured over the hermaphrodite flowers with paper clips or staples. When the anther sacs burst and self-pollination occurs in the flower, the fruit will develop inside the paper bag. It is not necessary to remove the bag to expose the developing fruit because when the fruit reaches a certain size, it will burst out of the bag. It usually takes about 135–150 days from bagging of flowers to fruit ripening and seed harvest.

4.11.1.2 Producing Seed of Cross-Pollinated Varieties

Some gynodioecious varieties like 'Sekaki' is considered cross-pollinated because the fruit do not set well or produce very little seed when self-pollinated. To reproduce seed of such varieties with good purity, the hermaphrodite flowers are bagged in the same way as described above. Next, pollen is collected from hermaphrodite flowers in the population. It is important to collect pollen from many trees so that the highly heterozygous genetic makeup and vigour of the variety is maintained. Pollination with pollen from too few trees will gradually result in narrowing of the gene pool due to genetic drift, and subsequently loss of varietal identity. Pollination is carried out when the hermaphrodite flower is freshly opened. The bag that covers the flower is removed temporarily and the pollen mixture introduced onto the stigma with a light brush. There is no necessity for emasculating the flower prior to pollination. The bag is replaced immediately after pollination. This same procedure applies for production of seed from dioecious applies for production of seed from dioecious varieties.

4.11.1.3 Producing Seed of F_1 Hybrid

F_1 hybrid seed like 'Eksotika II' are produced by hybridisation of the inbred parents 'Line 19' and 'Line 20'. Where there is no reciprocal differences in the cross, the male parent is the one that produces abundant pollen and the female should be very fruitful. In 'Eksotika II', 'Line 19' is used as the maternal parent because it does not produce pollen well due to high carpellody of stamens and sterility (Chan 1993). Production of hybrid seeds requires more effort because of the need to emasculate the hermaphrodite flowers prior to pollination. However, it is easier in papaya because of the large number of seeds that can be obtained from a single pollination. Prior to pollination, the selected unopened hermaphrodite flower is emasculated before the anther sacs dehisce. The corolla tube of the bud is gently opened to expose the stamens attached to the petal beneath the stigmatic lobes. A pair of fine forceps is used to remove the ten anthers. After emasculation, another pair of fine forceps is used to pick a freshly dehisced anther from the pollen donor parent and to gently rub it onto the stigma. The flower is covered with a wax paper bag immediately after the operation.

Hybridisation using female flowers will obviate emasculation and the seed production is also four times higher compared with using hermaphrodites (Chan and

Mak 1993b). However, seed developed from female fruit have a higher percentage of undesirable female trees and, for this reason alone, the higher cost in using hermaphrodites for hybrid seed production in papaya is justified.

4.11.2 Seed Treatment and Storage

Papaya seed can be harvested when the fruit reaches 'colour breaker' stage. The seed should not be harvested too late because a high percentage has been known to germinate within the fruit in some varieties.

Papaya seeds are non-recalcitrant and can be dried to moisture levels of 9–12% for long-term storage (Teng and Hor 1976). Seed freshly harvested from fruit have very low and variable germination. This is because the sarcotesta (Arumugum and Shanmugavelu 1975) and the seed itself (Yahiro and Hayashi 1982) contain growth inhibitors that prevent wasteful germination while the seeds are still in the fruit. Certain treatments must be done to the seed prior to storage or planting so that they can store longer as well as germinate with good viability and uniformity. Removal of the sarcotesta promotes germination considerably even in fresh, undried seed, but germination is further enhanced by seed drying and cool temperature storage at 15 °C (Yahiro 1979). The requirement of cool temperature to break dormancy in papaya is similar to vernalisation for temperate seeds; although the temperature for vernalisation is lower (5–10 °C). Yahiro and Hayashi (1982) reported that storage of papaya fruit for 30–50 days under 15 °C greatly reduced the activity of growth inhibitors in the seed, resulting in improved rate and uniformity of germination.

In the tropics, the practical methods for drying of papaya seed are either under the sun or air-drying in the shade. Chacko and Singh (1972), using the Washington variety, reported that there was no difference in germination using either method. Chan and Tan (1990), however, found significant interaction between four genotypes and drying treatments (sun or shade, with or without sarcotesta) for seed germination. Sun- or shade-drying, with or without sarcotesta, gave good germination for three of the varieties, but for 'Eksotika', sun-drying with the sarcotesta intact gave very poor results. Papaya seed without sarcotesta, well-dried and stored at a temperature of 5 °C can retain good germination of 60–70% even after 5 years of storage (Chan 1991).

4.12 Future Breeding Prospects

Papaya is an important tropical fruit in international trade and nearly all exported papayas are air-flown because of the delicate nature of the fruit and the short storage life. The prospects in market expansion for papaya in many countries are therefore restricted by the expensive and often limited air cargo space. There is little opportunity for expansion of export markets if technology does not permit the export of papaya by less expensive sea transport. Success in development of delayed ripening

in papaya will offer inroads to exploit this prospect. In papaya production, the dreaded PRSV and bacterial dieback diseases will continue to loom as threats to the industry unless resistant varieties can be developed to restore confidence of growers and investors. Solutions to these two technical constraints, namely, delayed ripening and PRSV resistance, are being aggressively pursued using primarily biotechnology as the tool. Although the progress of such work in the USA, Australia, Taiwan and Southeast Asian countries appeared encouraging with development of commercial transgenic varieties and advanced breeding lines, there is growing public dissent towards the development and release of genetically modified organisms to the environment. The other problem in this field of research is the license agreement signed with the technology owners, which may restrict the dissemination and export of the transgenic fruit. These factors will adversely affect or even completely ruin the export trade of papaya in the country. Growers and exporters may revert to non-transgenic varieties to tap the lucrative export markets in anti-GMO Europe and Japan than use the new transgenic papaya, even if they are far more promising in disease resistance and keeping quality.

Regarding future breeding objectives for papaya, it is evident that priorities will change in tandem with industry needs. The stepwise priorities in papaya breeding were discussed by Chan (2005) and are summarized in Fig. 4.7. Varieties were developed and improved in a stepwise manner according to the changing industry demands. Using the Malaysian breeding programme as an example, the early selection criteria were just uniformity and distinctiveness. Interim varieties like 'Subang 6' for table and 'Sitiawan' for processing were quickly recommended to jumpstart the industry. These early interim varieties did not have good fruit qualities. So the next rung of priorities include sugar content, flavour, flesh colour, firmness, cosmetics, varietal stability and performance quickly followed suit. The current priorities in extension of fruit storage and disease resistance will likely go into the next decade, after which designer traits such as enhancement of phyto-chemical and nutritional contents for the food, pharmaceutical and cosmetics industries and novelty in serving without the inconvenience of cutting (e.g. peeling fruit) will be given priority.

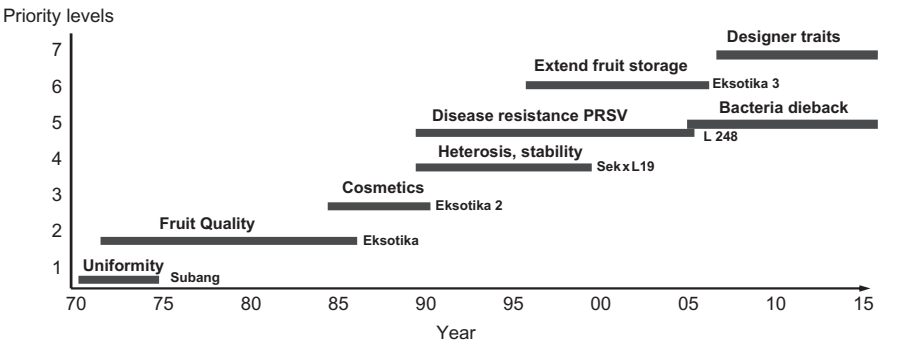

Fig. 4.7 Summary of stepwise priorities in papaya breeding programme and output of varieties in Malaysia

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Chapter 5
Grape Breeding

Phyllis Burger, Alain Bouquet, and Michael J. Striem

5.1 Introduction

Grapevine is one of the most widely grown crops in the world and covers about 7,955 million hectares. In 2003 the world wine production was 2,667 million hectolitres with France, Italy and Spain the leading producer countries. Table grape world production was in the order of 174 million metric ton. China is the leading producer country, but the bulk of the Chinese crop is consumed locally and little of the Chinese table grape industry is known to the Western world. The largest exporter of table grapes is Chile, followed by Italy (OIV statistics for 2003, http://www.oiv.int/). It is generally believed that *Vitis vinifera*, originated in the Middle East and that cultivation of the grapevine began during the Neolithic era (6,000–5,000 BC) along the eastern shores of the Black Sea in the region known as Transcaucasia in the areas south of the Caspian and the Black Sea (Mullins et al. 1992). From its centre of origin, the grapevine traveled with man to other parts of the world and in ancient Egypt mention was made of grapevine growing and wine-making. The domesticated grapevine reached European and North African Mediterranean countries, first with the Phoenician and Greek trade routes and later with the Romans, who spread it through their Empire. From Europe, the domesticated grape spread globally with settlers to the more temperate climates in the Americas, South Africa, Australia and New Zealand. *V. vinifera* grows in temperate climates and flourishes in Mediterranean climates, can be cultivated in tropical climates and with careful mangement and pruning may produce two crops a year (Possingham et al. 1990).

Of the 17 genera belonging to the family *Vitaceae*, that developed its own identity inside the Eudicots at the end of the Cretaceous period about 100 million years ago (Ingrouille et al. 2002), it is only the genus *Vitis* that produces edible fruits. Planchon (1887) divided the *Vitis* species between two sub-genera namely *Euvitis* (bunch grapes) and *Muscadinia* (muscadine grapes). *Muscadinia* has a genome of $2n = 40$,

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similar to the one of several other genera of *Vitaceae* (*Ampelopsis*, *Ampelocissus*, *Parthenocissus*), while for *Euvitis* it is $2n = 38$. Though some researchers (Olmo 1986; Mullins et al. 1992) argued that *Muscadinia* should be elevated to generic level as is supported by morphological, anatomical and karyological characteristics, it has not achieved general acceptance. *Muscadinia* is endemic to the southeastern states of the USA and among the three species known, only *Vitis rotundifolia* Michx. is of commercial value (Olien 1990).

Great genetic diversity is found in grapevines and they are adapted to different soils and climates. The majority of the table and wine grapes grown commercially are from *V. vinifera* origin. In the USA, some other *Vitis* spp. are grown commercially and were of local economic importance (Hedrick 1908). A number of American *Vitis* spp. were used in the past and are still being used in breeding programmes, especially in developing phylloxera resistant rootstocks, but also disease resistant wine and table grape cultivars. Of these, the following are of highest importance: *V. labrusca, V. aestivalis* and *V. rupestris Vitis* spp. native to tropical America, like *V. smalliana, V. caribaea* and *V. shuttleworthii* are included in breeding programmes to develop cultivars adapted to climatic conditions of the tropics (Camargo, 2000). In the Eastern European and Asian countries, *V. amurensis* is often included in breeding programmes to introduce cold hardiness and disease resistance.

Man's interest in grape growing evolved by selection from wild vines bearing fruit with desirable eating or other superior characteristics. The origins of many of the well-known wine and some table grape cultivars are still shrouded in mystery. They probably originated from chance seedlings or as sports of old cultivars and were easily propagated by cuttings and grown for centuries. Though the use of deliberate crosses was likely since the Middle Ages (Boursiquot et al. 2004), it is only since the late nineteenth century that such crosses are well-known. Some important wine grape cultivars were developed from these crosses (e.g. Alicante Bouschet in France that was bred by Louis and Henri Bouschet between 1829 to 1855 (Snyder 1937), Müller-Thurgau in Germany that was bred by Müller-Thurgau in 1882 (Dettweiler et al. 2000) and Pinotage in South Africa that was bred by Perold in 1925 (Orffer 1979)). Although grapes for wine-making were grown from antiquity, table grape production only became an important enterprise by the end of the nineteenth century (Einset and Pratt 1975).

5.2 Flower Types and Flower Structure

Oberle (1938) published a comprehensive study on flower types and the inheritance of floral morphology. He recognized three flower types: functionally hermaphroditic, functionally pistillate and functionally staminate and found that only one type of flower normally occurred on any one individual. In staminate types, which are functionally male, the pistil is incompletely developed and non-functional, while functionally pistillate or female types have non-functional, reflexed stamens. Perfect or hermaphrodite types have flowers in which both the stamens and pistils are functional. American species bear male and female

flowers on separate vines, while most European grape cultivars of *V. vinifera* origin bear hermaphrodite flowers (Snyder 1937; Olmo 1943). Various breeders postulated different hypotheses for the inheritance of flower type (Einset and Pratt 1975).

In perfect flowers, the pistil is normally surrounded by five stamens, although the number may vary from five to more on individual flowers of the same flower cluster. The fused corolla encloses the above-mentioned flower parts. At flowering it becomes loosened at the base and comes off like a cap (Snyder 1937). After fertilisation has taken place, the ovary develops into a seed in seeded cultivars or rudimentary seed in most of the 'seedless' types. For a review on anatomy and development of the reproductive organs of the grapevine, see Pratt (1971).

5.3 Flowering and Pollination

Grapevine is either self-pollinated or pollinated by wind, but honeybees and some other insects may also be active pollinators (Bronner and Wagner 1997). Olmo (1943) found honeybees to play an active role (at least in some seasons) in the cross-pollination of the table grape Almeria with reflexed stamens. Kimura et al. (1998) showed that insects contributed more than wind in the pollination of the dioecious *V. coignetiae*.

Not all the flowers on a bunch open simultaneously and some of the varieties are cleistogamous (self-pollination takes place before opening of flowers). The percentage of flowers in which cleistogamy occurred varied greatly among cultivars and appeared to be a genetic trait (Barbagallo et al. 1988; Staudt 1999). Heazlewood and Wilson (2004) also found that anthesis occurred before cap fall and found pollen to be viable, although pollen tube growth only started after cap fall. Furthermore, pollen remained viable for several days after cap fall. Bronner and Wagner (1997) found pollination to be highly influenced by temperature, relative humidity and stigma receptivity. Breeders may find the work of Staudt (1999) on flowering cycles, on the influence of temperature on pollen germination and the tempo of pollen tube growth (Staudt 1982) helpful in deciding on the best time of day for cross-pollination. Some fungicides and other chemicals sprayed in the vineyard against *Botrytis* may have an effect on pollen germination and pollen tube growth (Heazlewood et al. 2005).

5.4 Controlled Cross Breeding

Grape breeding is based primarily on hybridisation. Self-pollination as breeding method has been poorly worked out and most of the attempts were unsuccessful due to strong inbreeding depression. However, Pospisilova (1974) crossed inbred generations (I_1) of the *V. vinifera* cultivars Traminer and Veltliner and obtained higher productivity in the progeny compared to the cross between these two cultivars.

Bronner and Oliveira (1990) isolated from the sixth self-pollinated generation of Pinot noir, a homogeneous progeny, which they considered as a 'quasi pure line' usable for genetic studies. Finally, Todorov (2000) is the only breeder who obtained a commercial table grape cultivar (Velika) by self-pollinating a genotype selected in the progeny of a Bolgar x Ribier cross.

5.4.1 Emasculation and Pollination

Since the flowers of most of the commercial grape cultivars are hermaphroditic, it is necessary for breeders to emasculate flowers before self-pollination takes place. Emasculation is done by removing the cap and stamens, usually with a fine-point forceps (Fig. 5.1). Emasculated clusters are pollinated by hand and covered with paper (or other suitable) bags to prevent contamination by 'foreign' pollen. Some breeders use a small brush and dust stigmas with pollen (Snyder 1937), while others simply put a flowering cluster in the paper bag with the emasculated cluster and shake the bag or simply tap a flowering cluster of the chosen pollen parent against the emasculated cluster; Barrett and Arisumi (1952) described a home-made 'pollen atomizer' or 'duster' used for pollination.

5.4.2 Pollen Collection and Storage

Pollen can be collected in bags in the vineyard by covering clusters before anthesis and removing these after flowering or clusters may be harvested and dried indoors. In the latter case, clusters are collected when the first flowers start opening, taken indoors and stripped from the cluster and spread in a thin layer on glass sheets. These are placed in a warm, dry area free of air currents where pollen is left to dry for approximately 24 h. Dried flowers are scraped off the glass, rubbed through a sieve and pollen collected by scraping it off the glass plate into a suitable container (Barrett and Arisumi 1952). Equipment and hands need to be cleaned with alcohol to prevent contamination when working with more than one cultivar (Barrett and Arisumi 1952). Dried pollen are stored in a desiccator at −16 °C with silica gel (Bronner and Wagner 1997). These authors also found that germination percentages of pollen exposed to UV radiation rapidly decreased.

Breeders usually prefer to use pollen as soon as they are collected or within the same season, but under optimal conditions it may be stored for a limited number of years. It is advisable to do germination tests before using stored pollen. Olmo (1942) reported that pollen showing a germination percentage of as low as 6% gave as good a set in the field as fresh pollen. Bronner and Wagner (1997) found optimum temperatures for germination to be between 22 and 26 °C and added 20% sucrose to the medium as well as boric acid (5 mg/l) and agar (20 g/l). Agarwal (1983) used various organic solvents to store pollen at 4–6 °C. Ganeshan and Alexander (1990)

Fig. 5.1 Grape flowers (*top*) and emasculation of clusters (*below*)

obtained viable progeny when pollen stored for up to 64 weeks in liquid nitrogen was used in crosses, but seed yield was reduced after 5 years in storage.

5.4.3 Seed Germination

Grapevine seeds are dormant and therefore are stratified in moistness under controlled temperatures (usually around 4 °C) for 2–3 months, followed by germination at around 25 °C (see Einset and Pratt 1975 for references).

Researchers have investigated the use of chemicals to aid in seed germination. Spiegel-Roy et al. (1987) reported the efficiency of cyanamide in overcoming dormancy. Manivel and Weaver (1974) used various treatments and only gibberellic acid was partially effective. Ellis et al. (1983) proposed the following procedure for grapevine seed germination: a 24 h soak in H_2O_2 (0,5M) a further 24 h soak in 1,000 ppm GA_3, followed by a 21 day pre-chill at 3–5 °C with germination in a diurnal alternating temperature regime of 20–30 °C (16/8 h).

5.5 Breeding for Specific Characteristics

Characteristics most desirable vary depending on whether grapes are produced for winemaking, juice, fresh consumption, raisin production or for use as rootstocks for grape propagation. For table grapes, preferences differ between markets and countries. In general, these include seedlessness, large natural berry size, good eating quality and other unique characteristics. In some grape growing countries, cold hardiness is very important and for table grape producers in countries far from the world-markets, cold storage is extremely important. With high costs incurred to protect the crop from major fungal diseases and a higher awareness of environmental issues amongst consumers, the production of disease resistant cultivars that would also comply to the other criteria for table, wine and raisin grapes are very important.

5.5.1 Fruit Characteristics

5.5.1.1 Seedlessness

Worldwide, seedless table grapes are high in demand and, therefore, many breeders focus much of their efforts on the creation of new seedless cultivars. From a consumer's perspective, seedless grapes would be those with undetectable rudimentary seeds (seed traces), while from a botanical viewpoint only parthenocarpic cultivars would be truly seedless. Breeders regard seedlessness to be of two types. Seeds are never formed in the parthenocarpic cultivars like Black Corinth, but rudimentary seeds (which may contain viable embryos) develop in the stenospermocarpic cultivars like Sultanina and are the result of embryo and/or endosperm abortion. When parthenocarpic cultivars were used as pollinators of various seeded cultivars no seedless progeny was obtained (Stout 1937), while seedless progeny could be obtained when stenospermocarpic cultivars were used to pollinate seeded cultivars (Stout 1937; Weinberger and Harmon, 1964). It is thus clear that breeders would focus on the stenospermocarpic cultivars.

Rudimentary seeds vary in size, and lignification and classification of offspring into seedless and seeded individuals varied greatly amongst researchers. Some based their classification on detectability of the rudimentary seed (Loomis and Weinberger 1979; Spiegel-Roy et al. 1990a). Ledbetter et al. (1994) used sinker frequency, relative seed mass (sinker frequency multiplied by average sinker mass) and seed ratio (total seed weight divided by total fruit weight) as parameters. Ramming et al. (1990a) viewed 25 mg fresh weight as the division between seeded and seedless genotypes, but placed the maximum seed trace size for consumer acceptance around 10 mg fresh weight. Striem et al. (1992) used four categories for seed/seed trace size and regarded hardness of the seedcoat and the degree of endosperm development as sub-traits of seedlessness. Furthermore, the perceptibility of the seed traces was not necessarily correlated to size, but rather to the hardness of the seed-coat. To complicate matters, rudimentary seed size may differ from year to year and also seem to be influenced by vine age and rootstock (Christensen et al. 1983).

The seeded:seedless ratios in progeny of crosses between seeded and stenospermocarpic seedless cultivars varied greatly and, thus, led to various hypotheses to explain the inheritance of the stenospermocarpic trait. Weinberger and Harmon (1964) and Loomis and Weinberger (1979) regarded seedlessness to be controlled by complex recessive genes. Ramming et al. (1990a) supported this view and concluded the seedless trait not to be controlled by a single recessive gene, since all seedless × seedless crosses had some seeded offspring. Spiegel-Roy et al. (1990a) postulated two complementary recessive genes. The hypothesis of Bouquet and Danglot (1996) stated that inheritance of seedlessness was based on a complex system whereby the expression of three independently inherited recessive genes was controlled by a dominant regulator gene.

5.5.1.2 Other Fruit Characteristics

The heritability of a number of quantitative traits has been studied, including berry characteristics and ripening (Fanizza and Raddi 1973; Firoozabady and Olmo 1987; Eibach 1990; Wei et al. 2002).

Some breeders investigated the inheritance of berry skin colour, a relevant trait influencing both table grape and wine making quality. Barritt and Einset (1969) proposed two pairs of genes for fruit colour inheritance. A gene for black (blue) colour (*B*---) dominant, epistatic to that for red and white fruit and with red fruit (*bbR*-) dominant to white (*bbrr*). Recently Kobayashi et al. (2004) indicated that variation for berry skin colour is associated with the presence of a retrotransposon in the promoter region of a transcription factor that is involved in skin colouration and probably corresponds to the gene B.

Researchers also reported on the inheritance of aromas, mostly the *V. vinifera* muscat flavour. Wagner (1967) proposed that three to five complementary genes and a modifying gene were involved. Eibach et al. (2003) identified three aroma terpene compounds – namely Hotrienol, Trans-p-Linalooloxide and Terpendiol II – in offspring from a cross between a muscat and non-muscat parent. With regards to the *V. labrusca* (foxy) flavour, Reynolds et al. (1982) proposed a three-gene, dominant and complementary system for methyl anthranilate (MA) and a two-gene system for total volatile esters (TVE). Fisher et al. (1990) confirmed the previous hypothesis for TVE, but suggested a more complex environmentally influenced system for MA.

5.5.2 Resistance to Pests and Diseases

V. vinifera evolved in mild climates and without the presence of the economically most damaging pathogens, like the downy mildew (*Plasmopara viticola*), powdery mildew (*Uncinula necator* Syn. *Oidium tuckeri* and *Erysiphe necator*) and insects like phylloxera (*Daktulosphaira vitifoliae*). The *vinifera* grapevine is often very susceptible to these biological factors as it became clear when, for example, it was exposed to these stress factors in the USA. By the end of the nineteenth century, pests and diseases introduced from North America, caused havoc in the European

grapevine industry. Powdery mildew appeared in 1845 in France and by 1851 it had spread to all the vineyards in Europe. Phylloxera found its way to Europe shortly hereafter in 1865 and was followed by downy mildew (1878) and black rot (*Guigardia bidwellii*) in 1885 (Krul and Mowbray 1984).

5.5.2.1 The Use of Euvitis Species in Resistance Breeding

The introduction of American species resistant to phylloxera (mainly *V. riparia*, *V. rupestris* and *V. berlandieri*) and intensive breeding programmes saved grapevine production in many regions of the world (Pouget 1990). Among the resistant species, only *V. berlandieri* was adapted to the highly calcareous soils, but could not be used in its pure form and hybridisation was necessary to develop rootstocks resistant to lime-induced chlorosis. Rootstocks were also bred for resistance against nematodes like *Meloidogyne* spp. (Cousins et al. 2003) and *Xiphinema* spp. (Meredith et al. 1982).

Although chemical sprays (sulphur and copper) were rapidly effective against the fungal diseases, these are still difficult to control and costs are high. Very early, numerous European breeders made crosses between *V. vinifera* and resistant American *Vitis* spp. like *V. rupestris, V. labrusca, V. riparia* and *V. aestivalis* to combine their resistance with the fruit quality of *V. vinifera*. Although hundreds of so-called 'direct producer hybrids' were introduced to the French wine industry during the first half of the twentieth century, they are not of commercial importance today, but some of them (Villard blanc, Chambourcin, Seyval) were intensively used in modern breeding programmes for disease resistance (Kozma 2000; Eibach and Töpfer 2003). Resistance to powdery mildew was also found in the wild Chinese species *V. bryoniifolia, V. davidii* and *V. piasezkii* (Wang et al. 1995) and to downy mildew in the Asiatic species *V. amurensis* (Korbuly 2000). Other fungal diseases apart from the mildews, that breeders endeavour to develop resistance against, include anthracnose (Mortensen 1981) and *Botrytis*, while resistance against bacterial diseases include Pierce's Disease (PD), of importance in the USA (Mortensen 1968; Krivanek et al. 2005).

In *Euvitis*, Boubals (1959) postulated resistance to downy mildew to be dependent on two genic systems: a single gene for the hypersensitive reaction at the time of infection and several genes for the inhibition of growth of the fungal mycelium. Boubals (1961) postulated resistance to powdery mildew to be dependent on a polygenic system. Li (1993) also viewed resistance to powdery mildew to be of a polygenic nature and found minor resistance genes in *V. vinifera*. Eibach (2000) found different genes to be responsible for resistance to downy and powdery mildew and that no marked linkage seemed to exist between those genes.

5.5.2.2 The Use of Muscadinia in Resistance Breeding

Muscadinia is native to the southeastern United States and is a useful source for genes of resistance to phylloxera, nematodes, PD and fungal diseases. Often, these

genes have a high degree of dominance (Olmo 1986). This was confirmed by Bouquet (1983) who found a high degree of resistance to phylloxera in *Muscadinia*. Bouquet (1981) found the muscadines resistant to *Xiphinema index* the vector of grapevine fanleaf virus (GFLV), but not to the virus itself and also confirmed a large degree of dominance of this resistance (Bouquet et al. 2000a). By back-crossing a resistant F_1 hybrid to the rootstock cultivar 140 Ruggeri, a new rootstock resistant to virus spread has been selected (Bouquet et al. 2004). In *Muscadinia*, the same author also identified a dominant gene called *Run* 1 that confers resistance to powdery mildew and which he introduced in advanced back-cross progenies with *V. vinifera* (Bouquet, 1986; Bouquet et al. 2000b). It appeared that genotypes carrying the *Run* 1 gene also showed partial resistance to downy mildew due to the *Rpv* 1 linked gene (Merdinoglu et al. 2003). Current strategies being developed in European countries aim at combining genes of resistance from *Muscadinia* and *Euvitis* (Kozma and Dula 2003).

5.5.2.3 Resistance to Abiotic Stress

In breeding programmes for some wine grapes and rootstocks, tolerance to abiotic stress factors is also important. Rootstocks of *V. rupestris* × *V. berlandieri* ancestry were found the most tolerant to drought in greenhouse conducted tests by Carbonneau (1985). Pouget (1980) bred a new rootstock cultivar highly resistant to iron chlorosis by inter-crossing rootstocks of *V. vinifera* × *V. berlandieri* ancestry. Further work, using inter-crossing rootstocks of *V. riparia, V. rupestris* and *V. berlandieri* ancestry led to another new cultivar well adapted to acid soils (Pouget and Ottenwaelter 1986).

Cold tolerance is found in *V. riparia, V. labrusca* and *V. amurensis* (Reisch and Pratt 1996) and was largely used in breeding programmes for wine grapes in the USA (Hemstad and Luby 2000) and Eastern Europe (Korbuly 2000). For discussion on resistance breeding against other fungi, bacteria, insects and abiotic stress factors, see Mullins et al. (1992) and Reisch and Pratt (1996).

5.6 Tissue Culture Techniques

The value of conventional methods in grapevine breeding is limited by long generation intervals, the highly heterozygous nature of the vine and inbreeding depression. Furthermore, few traits of viticultural importance are controlled by single genes with dominant alleles. Thus, researchers have embarked on investigating biotechnological tools in grapevine improvement. Tissue culture techniques are used for micro-propagation by shoot tip culture, production of virus-free clones, protoplast culture, embryo rescue, organogenesis, callus induction, somatic embryogenesis and genetic transformation.

5.6.1 Micro-propagation

In practice, micro-propagation is mostly applied for the production of virus-free plant material. The technology for micro-propagation of shoot tip culture is well established and the resulting plants appear to be genetically identical to the mother plant. According to Bouquet (1989) the role of tissue culture in commercial propagation is likely to be limited because standard propagation systems based on grafting are well established. However, this author identified five possible situations where tissue culture could be of benefit: (1) Rapid multiplication of newly bred or imported cultivars, rootstocks in particular, (2) propagation, maintenance and international exchange of virus-free plants, (3) maintenance of germplasm under slow-growth in vitro conditions, (4) application of thermotherapy to obtain virus-free plants and (5) testing in vitro for resistance to biotic and abiotic stress factors.

This chapter will not deal with in vitro multiplication and micro-grafting techniques and their use in the production of virus-free material, since reviews were published elsewhere (Krul and Mowbray 1984; Monette 1988; Gray and Meredith 1992; Reisch and Pratt 1996; Torregrosa et al. 2001; Bouquet and Torregrosa 2003). These publications also address other biotechnological techniques not discussed here or only touched on, like organogenesis, haploidy induction, etc.

5.6.2 Embryo Rescue

The development of embryo rescue techniques enabled breeders to develop offspring from two stenospermocarpic seedless parent cultivars. Although labour intensive, these techniques are applied routinely in the majority of table grape breeding programmes as the proportion of seedless progeny in such crosses are much higher than in conventional seeded × seedless crosses. Cain et al. (1983) reported the first successful embryo rescue of grapevine, followed soon after by Emershad and Ramming (1984) and Spiegel-Roy et al. (1985).

Genotypes, media composition, culture dates (days after pollination), effect of cold treatments and various interactions were studied in numerous publications. For instance, Gray et al. (1987) compared the effect of liquid medium to that of solid medium. In general, higher germination rates were obtained when rudimentary seeds were cultured at a later date, though some researchers found no or little correlation. Correlations between size of the rudimentary seed and the presence of viable embryos were found by Bouquet and Davis (1989) and Spiegel-Roy et al. (1990b). Although embryos are able to germinate directly from the rudimentary seeds, researchers found it necessary to dissect rudimentary seeds after two to three months in culture and remove the embryos for maximum recovery of plants (Cain et al. 1983; Ramming et al. 1990b). Gray et al. (1990) reported that embryos appeared to be dormant. Varying results were obtained when cold stratification and plant growth regulators were investigated to break dormancy of embryos (Emershad and Ramming 1984; Bouquet and Davis 1989; Gray et al. 1990). Seed

from early ripening seeded cultivars usually have low germination rates and embryo rescue techniques were successfully applied to develop seedlings from such cultivars (Ramming et al. 1990b; Goldy et al. 1989).

Since the seedless trait is only found in *Euvitis*, embryo rescue techniques were also employed to develop plants from crosses between *Euvitis* and *Muscadinia*. Although it is difficult to regenerate progeny from such crosses, Ramming et al. (2000) reported the first *V. vinifera* × *V. rotundifolia* seedless seedling. Some researchers investigated the application of chemicals in the vineyard to aid in embryo rescue. Kender and Remaily (1970) were able to produce viable seeds from seedless cultivars by applying ethephon. Bharathy et al. (2003) found pre-bloom and bloom sprays of BA beneficial for in vitro embryo and plant recovery, while Ponce et al. (2002) found putrescine to increase the number of embryos.

5.6.3 Screening for Resistance to Biotic Stress

Classical selection methods include screening of plants in vineyards or greenhouses. Aldwinckle (1978) found a correlation between field resistance and plants selected in greenhouses for powdery mildew resistance. However, Eibach (1994) found that powdery mildew infection on leaves and berries may vary considerably and that screening for resistance by using leaves was not necessarily applicable to berries. Leaf discs were also used to evaluate various accessions of *Vitis* spp. and hybrids for downy (Staudt and Kassemeyer 1995) and powdery mildew (Peros et al. 2006). Stein et al. (1985) found a good correlation between the development of powdery mildew on leaf discs and on vines in the field and the greenhouse. With downy mildew the correlation was not as good.

In vitro dual culture gives researchers the opportunity to study host/parasite interactions without the interference of environmental factors. Heintz et al. (1985) and Klempka et al. (1984) developed in vitro dual culture methods for powdery mildew and *V. vinifera* cultivars, while Sparapano et al. (2001) determined grapevine susceptibility to esca-associated fungi in micro-propagated shoots and callus. Following earlier studies, Bessis et al. (1992) suggested the use of phytotoxic polysaccharides, produced by *Botrytis* to select in vitro for resistance. However, Fanizza et al. (1995) found a low correlation between the response of in vitro assayed cultivars to *Botrytis* and bunch susceptibility under field conditions.

Mauro et al. (1988) found that the fungus *Eutypa lata*, which causes eutypa dieback (dead arm disease), produced toxic metabolites that induced typical symptoms on in vitro grapevines. Following this research, Soulie et al. (1993) developed in vitro tests to screen in vitro propagated plants and micro-cuttings of *V. vinifera* cv. Ugni blanc for tolerant clones. Jayasankar et al. (2000) selected plants resistant to anthracnose by exposing embryogenic masses of *V. vinifera* cv. Chardonnay to culture filtrate of the fungus.

Techniques to screen potential parent cultivars and seedling populations include phytoalexin production and the stilbene oligomers, ε-viniferin and α-viniferin,

which were found to be closely associated with resistance to downy mildew (Langcake 1981; Dercks and Creasy 1989) and also *Botrytis* (Sbaghi et al. 1995). However, Barlass et al. (1987) found the production of the phytoalexin precursor, resveratrol, highly sensitive to environmental changes and its usefulness for screening limited. Kortekamp and Zyprian (2003) found a correlation between peroxidase activity and resistance to downy mildew.

Vitis and *Muscadinia* spp. were grown in in vitro dual culture with phylloxera by Forneck et al. (1996) and Grzegorczyk and Walker (1998) to study grapevine/phylloxera interaction. Kellow et al. (2002) confirmed the value of in vitro screening for resistance and determination of phylloxera biotypes. Van Mieghem and Goussard (1987) induced reproduction of the nematode *Meloidogyne javanica* on in vitro Chenin blanc plants, while Franks et al. (2003) could clearly distinguish between resistant and susceptible cultivars when grown in vitro in dual culture with *M. javanica*. Bavaresco and Walker (1994) described techniques for the in vitro dual culture of grapevine and *X. index*.

5.6.4 Screening for Resistance to Abiotic Stress

In vitro techniques were developed by Bavaresco et al. (1993) and Netzer et al. (1991) to screen grapevines for tolerance to lime-induced chlorosis. Apart from lime-chlorosis, salt-tolerant rootstocks are also important as the grapevine is easily affected by salinity. Lebrun et al. (1985) used single cells for in vitro selection of tolerance in *V. rupestris*. Barlass and Skene (1981) and Troncoso et al. (1999) found in vitro techniques suitable for the selection of salt tolerance in various rootstocks. However, Skene and Barlass (1988) stressed the need for verification under field conditions. Some rootstocks are known to be susceptible to magnesium deficiency and Bouquet et al. (1990) investigated the possibility of in vitro selection.

5.6.5 Somatic Embryogenesis

Somatic embryogenesis is the process of embryo initiation and development from cells that are not the direct product of gametic fusion and is of great importance in the improvement of grapevines. Researchers have used these techniques for the development of in vitro screening systems for resistance to stress factors, virus elimination, germplasm conservation and introduction of foreign genes by genetic transformation. Various organs, for example, unfertilised ovaries, flower clusters, anthers, tendrils, petioles and leaves from greenhouse or in vitro cultured plants, were used as explants to initiate callus. Though the response varied greatly, embryogenic cultures were established for various grapevine cultivars and species. The first reports of somatic embryogenesis were made by Mullins and Srinivasan (1976) who obtained plants via somatic embryogenesis from unfertilised ovules of Cabernet Sauvignon (*V. vinifera*) and Hirabayashi et al. (1976) who employed anther culture to develop

shoots from *V. thunbergii*. Since these early reports, numerous studies reporting successful somatic embryogenesis or organogenesis and plantlet formation were published. Successful plantlet development via somatic embryogenesis was achieved in many *Vitis* spp. and inter-species hybrids. For a review see Martinelli and Gribaudo (2001), Bouquet and Torregrosa (2003) and also references mentioned under genetic transformation.

The most recent and spectacular application of somatic embryogenesis in grapevine was the separation of the chimaeric periclinal L_1 and L_2 layers in *V. vinifera* Pinot meunier and the obtention of plants with phenotypes different from the original plant (Franks et al. 2002). Pinot meunier has a L_1 cellular layer responsible for its characteristic hairiness and also carrying a mutation in a gene homologous of the *Arabidopsis GAI* (Gibberellin inhibitor) gene. The mutation of this gene gives the plant a dwarf phenotype in which all the tendrils are converted into inflorescences, capable of flowering and bearing fruit after a few months (Boss and Thomas 2002). These characteristics confer considerable interest in future grapevine genetics and breeding on this genotype.

5.6.6 Protoplast Culture

A number of researchers studied protoplast culture, but encountered difficulties to regenerate plants. The first report of success was from Reustle et al. (1994) who regenerated plants from protoplasts derived from embryogenic material of the hybrid cultivar Seyval blanc. Zhu et al. (1997) obtained plants through somatic embryogenesis from protoplasts of cv. Koshusanjaku (*V. vinifera*). See also Papadakis et al. (2001) for a review on protoplast technology in grapevine.

5.6.7 Somaclonal Variation

In the wine grape industry, where many of the most prominent cultivars are of ancient origin, somatic mutations have undoubtedly taken place over the long period of vegetative propagation as can be seen in the many clones of established cultivars that were selected and are cultivated today. This somatic heterogeneity provides sources of variation that could be exploited by researchers to increase variability in existing cultivars, thus thwarting genetic erosion induced by clonal and sanitary selection (Bouquet 1989) and select for specific traits, particularly for resistance to biotic and abiotic stress. Schneider et al. (1996) presented the first molecular evidence for somaclonal variation in grapevine. Three plants with altered phenotype derived from protoclones of Seyval blanc showed modified RAPD profiles. Popescu et al. (2002) confirmed somaclonal variation in anther-derived grapevines at the molecular level by using AFLP (Amplified Fragment Length Polymorphism) techniques. See Predieri (2001) for a review of somaclonal variations and in vitro induced mutagenesis and see also Torregrosa et al. (2001).

5.6.8 Genetic Transformation

It is not possible to introduce specific characteristics or desirable traits into an existing cultivar by hybridisation without destroying its originality. Therefore, the alternative approach of using direct gene transfer is very promising for grapevine improvement. Researchers need to be able to select transformants from non-transformants and to confirm whether stable introduction of a foreign gene into *Vitis* has taken place. Most commonly the *npt*II and *hpt* genes conferring antibiotic (kanamycin or hygromicin) resistance have been used as selectable marker genes, while the *uid*A gene conferring β-glucuronidase (GUS) expression and the *gfp* gene conferring green fluorescence were used to confirm transformation. However, the use of antibiotics in the selection process is not widely accepted by the public. Reustle et al. (2003) investigated the possibilities of using mannose instead of antibiotics in a selectable marker system. However, none of the regenerated plants was transgenic. These results were confirmed by Kiefer et al. (2004) who found mannose as well as xylose unsuitable for use in selection systems for transformation.

Though some researchers use efficient biolistic (micro-projectile bombardment) transformation systems (Vidal et al. 2003), the majority make use of the *Agrobacterium*-mediated transformation system. This technology is based on the ability of *Agrobacterium* to insert genes into plant cells during the infection process. Although *A. tumefaciens* causes crown gall in grapevine, disarmed strains that do not induce crown gall are used for genetic transformation. The most suitable plant material for co-cultivation with *Agrobacterium* is embryogenic cell lines, of which the quality and developmental stage have a strong effect on transformation efficiency. The first report of researchers who obtained transformed plants were from Mullins et al. (1990) with *V. rupestris* 'St. George'. Please see Perl and Eshdat (1998) and Bouquet et al. (2006) for techniques and protocols for genetic transformation in grapevine.

5.6.8.1 Rootstock Transformation

GFLV is transmitted by the nematode *X. index*. Dangerous chemicals are used for soil disinfection and, therefore, many researchers put their efforts into producing transgenic plants with coat protein mediated protection. Krastanova et al. (1995) obtained *V. rupestris* and 110 Richter plants transformed with the coat protein of GFLV, while Mauro et al. (1995) obtained 41B and SO_4 plants also transformed with GFLV-CP. From 18 independent transgenic grapevine lines established in a naturally infected vineyard, 3 did not show reaction to GFLV infection 3 years after planting (Vigne et al. 2003). Bouquet et al. (2003a) found that transgenes (*npt*II, *uid*A and GFLV-CP), introduced into 110 Richter and *V. rupestris* du Lot rootstocks and transmitted by hybridisation in *X. index* resistant rootstocks developed by conventional cross-breeding, expressed normally with a mendelian segregation in offspring.

Le Gall et al. (1994) transformed 110 Richter with the coat protein of Grapevine Chrome Mosaic Virus (GCMV-CP). Torregrosa and Bouquet (1997) co-inoculated in vitro grown plantlets of the rootstock Gravesac with a mixture of wild

A. rhizogenes and *A. tumefaciens* carrying plasmids containing GCMV-CP genes. Transformed hairy root cultures were initiated from excised root tips. Plant regeneration was not achieved, but the authors mentioned the possibility to graft in vitro transgenic roots to non-transformed shoot systems. Radian-Sade et al. (2000) cloned the gene encoding the coat protein of Grapevine Virus A (GVA-CP) and used this gene to transform the rootstock 41B and tobacco. Martinelli et al. (2002) reported stable transformation of *V. rupestris* with the movement protein of GVA.

Guillen et al. (1998) purified a NADPH-dependent aldehyde reductase from *Vigna radiata* that converts eutypine, the toxin that is involved in eutypa dieback, into non-toxic eutypinol. Grapevine (*V. vinifera*) cells transformed with the gene (*Vr-ERE*) encoding the eutypine-reducing enzyme showed in vitro resistance to the toxin. Transformed plants were established only for the rootstock cultivar 110R (Legrand et al. 2003) and were not affected by relatively high concentrations of eutypine, whereas growth of untransformed plants were highly inhibited.

5.6.8.2 Scion Transformation

Mauro et al. (1995) reported the first transformed plants of a scion cultivar (*V. vinifera* cv. Chardonnay) with a gene of agricultural value, namely the GFLV-CP gene, while Gambino et al. (2005) reported transformation of cv. Nebbiolo with the same gene. Scorza et al. (1996) reported that by combining particle bombardment of somatic embryos with *Agrobacterium* co-cultivation produced Thompson seedless plants transformed with the lytic peptide Shiva-1 or the tomato ringspot virus (TomRSV) coat protein genes.

Chitinase is one of the hydrolytic enzymes, which can degrade fungal cell wall components. Because of this characteristic, hydrolytic enzyme coding genes are very attractive for researchers in their efforts to improve disease resistance. Neo Muscat (*V. vinifera*) plants transformed with a rice chitinase gene showed enhanced disease resistance to powdery mildew and anthracnose (Yamamoto et al. 2000). Harst et al. (2000) obtained Riesling plants transformed by antifungal genes (glucanase and chitinase), while Bornhoff et al. (2005) obtained transgenic Seyval blanc plants carrying genes for chitinase and RIP (ribosome inactivating protein). Although the foreign DNA was stably integrated, there was no visible improvement of field resistance against downy and powdery mildew. Agüero et al. (2005) obtained Chardonnay and Thompson Seedless plants transformed with the pear polygalacturonase inhibiting protein (pPGIP) gene. Plants were evaluated for tolerance to PD and *Botrytis* and delay in the development of PD was observed in some transgenic lines with increased pPGIP activity. Chardonnay plants, transformed with an antimicrobial peptide gene by using biolistics, were evaluated for resistance to crown gall and powdery mildew (Vidal et al. 2006).

Mezetti et al. (2002) used a novel approach based on organogenesis to introduce the gene *DefH9-iaaM* that conferred parthenocarpic fruit in tomato, eggplant, strawberry and raspberry in grapevine. See also Perl and Eshdat (1998), Thomas et al. (2000), Kikkert et al. (2001), Martinelli and Mandolino (2001), Colova-Tsolova

et al. (2001) and Bouquet et al. (2003b) for applications and prospects of genetic transformation in grapevine.

5.7 Molecular Studies

Since about fifteen years, the availability of inexpensive and easy-to-use molecular markers has considerably facilitated research in *Vitis* genetics and breeding. It is now possible to create unique DNA profiles for each genotype, to map the grapevine genome, to tag specific genes for breeding purposes, marker-assisted selection and gene cloning. For a general review on molecular markers, see Reisch (2000).

5.7.1 Fingerprinting and Diversity Assessment

Cultivar naming is a major problem in viticulture. Often the same cultivar is grown under different denominations or different cultivars are sometimes grown under the same denomination. Molecular markers have been widely applied in grapevine for cultivar identification. They include successively random fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) and single sequence repeats (SSR), also called micro-satellites. Standard sets of micro-satellite markers have been proposed (This et al. 2004) and they are now commonly applied to solve problematic naming (Dangl et al. 2001), for genetic diversity assessment (Sefc et al. 2000) and for parentage analysis (Bowers et al. 1999). For a review on micro-satellite markers, see Sefc et al. (2001). Other techniques for DNA fingerprinting are currently in development, such as retrotransposon-based molecular markers (Pelsy et al. 2003) or single nucleotide polymorphisms (SNP) (Salmaso et al. 2004).

Bowers and Meredith (1997) determined the parentage of Cabernet Sauvignon, while Bowers et al. (1999) investigated the origin of Chardonnay, Gamay noir and other important cultivars. Boursiquot et al. (2004) identified a number of synonyms for Gouais. Though not of importance today, this cultivar was widespread in Europe in the past and the authors underline the important role it played in the parentage of many of modern-day prestigious French and European cultivars.

However, the relationships between cultivated vines and wild vines have not yet been clearly established. Some results indicate the absence of links (This et al. 2001; Carreño et al. 2004). In contrast, other results suggested that local domestication of wild vines predominated over introduction of cultivars from other regions (Sefc et al. 2003; Grassi et al. 2003). Vine domestication has resulted in a radical change in vine biology, such as the change from dioecism to hermaphroditism. In their wild state, due to a strict allogamy, all the species of the *Vitis* genus are dioecious and, therefore, have a high level of heterozygosity that meets in cultivars or wild populations of *V.vinifera* and is revealed by micro-satellite studies (Sefc et al. 2000; Aradhya et al.

2003). The high heterozygosity of *V. vinifera* cultivars was also confirmed by the first studies investigating SNPs (Salmaso et al. 2004).

5.7.2 Genetic Mapping and Marker-Assisted Selection

Suitable screening methods for fruit quality and disease resistance are necessary for maintaining efficient breeding programmes. Some of these screening techniques were already discussed, but one of the most exciting techniques is the fairly recent development of Marker Assisted Selection (MAS). These techniques enable breeders to make sophisticated decisions regarding which parents to choose for crosses and also to pre-screen seedling populations for certain traits and discard those individuals with the undesirable characteristic, before establishment in the vineyard. Strong linkage between molecular markers and the genes (alleles) responsible for the expression of these traits has to be established. Genotyping the gene-pool of potential parents and identifying those carrying the desired alleles to be combined would enhance the efficiency of the breeding process.

Since many agronomical important traits are quantitatively inherited in grapevine, it is difficult to control them in breeding programmes. By establishing associations of these traits with linked molecular markers, molecular maps may be produced and genetic factors involved localized as quantitative trait loci (QTL). Once the correlation between a specific phenotype and molecular marker has been established, the inheritance of a trait can be scored in the progeny at very early stages of plant development. To find good correlation between molecular markers and the measured trait, a combination of several markers will be necessary (Striem et al. 1996).

In 1997, grapevine researchers founded the International Grape Genome Programme (IGGP) (http://www.vitaceae.org). Among the interests of the IGGP was development of a reference linkage map resulting from individual mapping projects and as a resource for physical mapping. Such a map is also useful for targeting genomic regions for more intensive mapping efforts, such as for localizing QTLs or for gene cloning. Researchers combined various markers in developing genetic maps, using essentially and successively RAPD, AFLP, and SSR markers. Twenty research groups of 10 countries worked cooperatively in the Vitis Microsatellite Consortium (VMC) to develop a large number of micro-satellite markers. Several linkage maps were constructed from inter-specific hybrid populations (Lodhi et al. 1995; Dalbó et al. 2000; Grando et al. 2003; Fischer et al. 2004; Doucleff et al. 2004; Lowe and Walker 2006) or *V. vinifera* populations (Doligez et al. 2002; Riaz et al. 2004 and Adam-Blondon et al. 2004). An integrated SSR map based on five mapping populations was recently published (Doligez et al. 2006).

Several large-insert comprehensive genomic libraries have also been developed for the grape genome using the bacterial artificial chromosome system (Tomkins et al. 2001; Adam-Blondon et al. 2005). The connection between genetic maps and a physical map based on a Cabernet Sauvignon BAC library is currently under way (Lamoureux et al. 2006).

5.7.2.1 Mapping for Seedlessness and Other Berry Characteristics

Striem et al. (1994; 1996) described a number of sub-traits of seedlessness and found RAPD markers with significant effects on several of these sub-traits. These authors could exclude most of the seeded individuals of the progeny by using a two-step process of marker assisted selection. As was already discussed, Bouquet and Danglot (1996) proposed three complementary recessive genes regulated by a dominant inhibitor gene, which they identified as the *Sd*I gene. This work was followed-up by a number of publications on marker assisted selection for seedlessness (Lahogue et al. 1998; This et al. 2000; Adam-Blondon et al. 2001; Doligez et al. 2002). Lahogue et al. (1998) used a bulk segregant analysis with RAPD markers, and developed a SCAR (sequence characterised amplified region) marker linked to the *Sd*I gene. This marker (SCC8) explained a large part of the phenotypic variation of seedlessness components traits. Doligez et al. (2002) constructed parental and consensus genetic maps from a F_1 population derived from a cross between two rudimentary seedless genotypes. They found QTLs for several sub-traits of seedlessness and berry weight. In an independent study Meija and Hinrichsen (2003) developed another highly assertive SCAR marker potentially useful to assist selection for seedlessness.

The gene controlling sex in grapevine was placed by Dalbó et al. (2000) on a linkage group corresponding to the LG 2 of the *V. vinifera* consensus map. Placement of the sex locus was confirmed by Lowe and Walker (2006). A major gene for berry colour was also placed on LG 2 (Doligez et al. 2002). Fischer et al. (2004) identified QTLs for véraison (onset of berry ripening) and berry size. Fanizza et al. (2005), using a table grape progeny Italia (seeded) × Big Perlon (seedless), detected QTLs for different components of fruit yield such as berry and cluster weight. Marino et al. (2003) detected several QTLs for free aroma volatile components on the map derived from the cross *V. vinifera* Moscato Bianco × *V. riparia* (Grando et al. 2003). Eibach et al. (2003) identified QTLs for three aroma terpene compounds in a progeny from a cross between a muscat and non-muscat parent.

5.7.2.2 Mapping for Disease Resistance

As mentioned Bouquet (1986) introduced powdery mildew resistance from *Muscadinia* into *V. vinifera* and found resistance to be conferred by a dominant allele at a single locus designated *Run*1. Pauquet et al. (2001) developed genetic markers tightly linked to the *Run*1 locus. Donald et al. (2002) described the mapping of resistance gene analogs (RGAs) to the *Run*1 locus. Barker et al. (2005) using a bacterial artificial chromosome (BAC) library described the construction of comprehensive genetic and physical maps spanning the *Run*1 locus, that enabled cloning of the resistance gene. Fischer et al. (2004) developed a map from progeny of a cross between a disease resistant and a susceptible cultivar. Regent, derived from the French hybrid Chambourcin, has a complex parentage, involving a number of wild *Euvitis* species and shows high field resistance to powdery and downy mildews, while Lemberger is susceptible. The authors found that resistance to the two fungal

pathogens relied on independent genetic factors. One major QTL region was identified for powdery mildew resistance, while a major QTL and a less pronounced "minor" QTL for resistance to downy mildew were found in Regent. In another preliminary study, Zyprian et al. (2003) constructed a partial map by using two downy mildew resistant parents (Villard blanc and Ga-47-42, derived from the French hybrid Seyval). The authors postulated that resistance to downy mildew in the two segregating populations was determined by different genetic factors. Marino et al. (2003) also identified two different QTLs for resistance to downy mildew in the *V. vinifera* × *V. riparia* map constructed by Grando et al. (2003).

Dalbó et al. (2001) constructed a genetic map from inter-specific cultivars and developed a marker for powdery mildew resistance. Doucleff et al. (2004) constructed linkage maps from a cross between two half-sib genotypes *V. rupestris* × *V. arizonica* that carry resistance to Pierce's Disease (PD) and *X. index*. A primary resistance gene to PD, *Pdr1* was identified and mapped by (Krivanek et al. 2006). Lowe and Walker (2006), using a progeny from a cross between Ramsey (*V. champini*) and Riparia Gloire (*V. riparia*) developed the first linkage map of rootstocks and presented it as a valuable tool for studying the genetics of many rootstock traits including nematode resistance, lime and salt tolerance, and ability to induce vigor.

5.8 Conclusions

Biotechnological techniques made great impact on grapevine improvement in the recent past, especially the past decade by the development of successful genetic transformation systems and stable insertion of foreign genes of agricultural importance into grapevine. The use of molecular markers and the creation of genetic maps have contributed to a better understanding of grapevine genetics. Though biotechnological and molecular tools are making a big impact on the efficiency of breeding programmes, the grapevine community should appreciate the great history and culture of the grapevine.

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Chapter 6
Breeding Date Palm

Ismail El Hadrami and Abdelbasset El Hadrami

6.1 Introduction

Date palm, *Phoenix dactylifera* L., is a perennial long-lived dioecious monocotyledon of great socio-economic importance especially in North Africa and the Middle East. These countries grow 62 million of the 105 million trees available worldwide on an area of over a million hectares (Fig. 6.1; Table 6.1). These 'trees' are cultivated not only for their valuable fruits (dates), but also for producing fuel, fibre and as shelter for ground crops. Production of dates is of approximately 6.5 million metric tons around the world (Table 6.2) and generates an important commercial activity. Countries such as Egypt, Islamic Republic of Iran and Saudi Arabia represent the top three producers worldwide. Furthermore, in the areas where it is cultivated, the date palm contributes to the creation of a micro-climate that enables agricultural development of other species.

6.1.1 History, Botany and Ecology

Date palm is considered as one of the oldest fruit trees domesticated by man and is mentioned in the Qur'an ('Shake the trunk of the palm tree towards thee: it will drop fresh, ripe dates upon thee. Eat then drink, and let thine eye be gladdened'. Qur'an 19: 25–26) as well as in the Bible ('The next day the great crowd that had come to the festival heard that Jesus was coming to Jerusalem. So they took branches of palm trees and went out to meet him, shouting "Hosanna! Blessed is the one who comes in the name of the Lord – the King of Israel!"' St. John 12: 12–13). The representations of this tree appear in hieroglyphic engravings of old Egypt like in the writings of Neolithic civilizations of Mesopotamia. Probably the earliest wild findings of date palm were recorded around 5000–6000 B.C. from Iran, Egypt and

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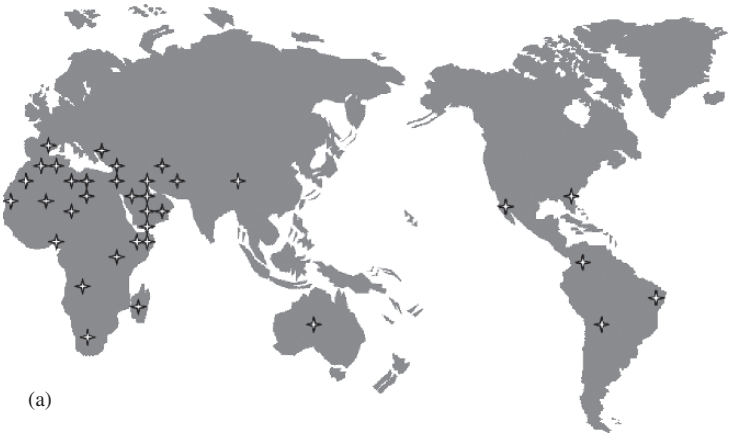
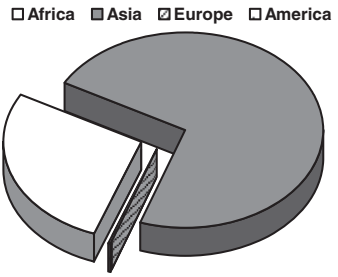


(a)



(b)

Fig. 6.1 Geographical repartition of date palm growing areas (a) and the relative groves surface per continent in Ha (2004) (b)

Pakistan while the earliest cultivations were found around 4000 B.C. from Eridu and Lower Mesopotamia. It had also been mentioned in Akkadian and Sumerian cuneiform sources dated 2500 B.C. and later. Date palm had been recorded in old history at the areas extending from the Indus valley (now Pakistan) to Mesopotamia in the Tigris/Euphrates valleys (now Iraq), the Nile valley, Southern Persia to the Eastern Mediterranean. Its centre of origin is still uncertain even if there are several claims that date palm originated from Babel in Iraq, from Dareen or Hofuf in Saudi Arabia or Harqan and also from an island on the Arabian Gulf in Bahrain. The oldest radiocarbon dated discovery of date seeds was on Dalma island, part of the Abu Dhabi Islands group. Two seeds were found in 1998, the oldest was 5110 B.C. and the other, 4670 B.C. It had been then introduced into Spain by the Moors and by the Spanish into the Americas. *P. dactylifera* is now found in tropical and subtropical regions all over the world as well as in temperate and arid regions in USA, Australia, southern Spain and the Mediterranean coast of Africa and West Asia.

The wild stock for the first domesticated variety was thought to originate in the southern near east of the fertile crescent (Zohary and Spiegel-Roy 1975). However, there are many other *Phoenix* species that have probably hybridised with the

Table 6.1 Expansion of the surface area of date palm groves around the globe over the last six years (FAO Stat 2004)

	Area occupied by date palm trees (Ha)					
	Years					
	1999	2000	2001	2002	2003	2004
Africa	268, 155	265, 702	304, 772	319, 379	334, 300	339, 305
Algeria	100, 120	100, 120	120, 036	135, 059	135, 000	135, 000
Benin	360	360	360	360	360	360
Cameroon	95	100	105	110	110	115
Chad	7, 600	7, 600	7, 600	7, 600	7, 600	7, 600
Egypt	28, 195	28, 982	29, 461	29, 620	29, 600	29, 600
Kenya	345	330	330	330	330	330
Libyan Arab Jamahiriya	23, 000	24, 000	28, 000	28, 000	28, 000	28, 000
Mauritania	5, 000	5, 000	8, 000	8, 000	8, 000	8, 000
Morocco	44, 200	30, 400	33, 600	33, 000	48, 000	48, 000
Niger	2, 200	2, 200	2, 300	2, 300	2, 300	2, 300
Sudan	26, 040	35, 000	35, 000	35, 000	35, 000	35, 000
Tunisia	31, 000	31, 610	39, 980	40, 000	40, 000	45, 000
Asia	639, 908	666, 025	660, 600	662, 575	665, 600	886, 430
Bahrain	830	823	823	1, 670	1, 670	1, 650
China	6, 000	6, 000	6, 000	6, 000	6, 500	7, 500
Islamic Republic of Iran	177, 272	184, 725	183, 269	184, 000	184, 000	185, 000
Israel	1, 301	2, 070	2, 170	2, 600	2, 600	2, 600
Jordan	251	264	264	346	554	550
Kuwait	1, 050	1, 350	1, 350	1, 350	1, 350	1, 400
Oman	35, 500	35, 508	33, 919	33, 869	33, 848	34, 000
Pakistan	76, 900	78, 590	78, 469	77, 900	78, 000	80, 000
Palestine	346	368	378	424	476	480
Qatar	1, 366	1, 343	1, 516	1, 463	1, 500	1, 500
Saudi Arabia	141, 750	142, 450	139, 099	139, 979	141, 421	145, 000
Syrian Arab Republic	1, 000	1, 009	1, 037	433	900	900
Turkey	3, 850	3, 440	3, 850	3, 850	3, 850	3, 850
United Arab Emirates	170, 330	185, 330	185, 330	185, 329	185, 330	186, 000
Yemen	22, 162	22, 755	23, 126	23, 362	23, 601	23, 600
Europe	525	754	856	764	856	856
Spain	525	754	856	764	856	856
America	2668	2915	2993	2787	3090	2997
United States of America	1, 983	1, 943	1, 983	1, 942	2, 104	2, 000
Mexico	617	894	926	759	900	900
Peru	68	78	84	86	86	97
World	911, 256	935, 396	969, 221	985, 505	1, 003, 846	1, 229, 588

Table 6.2 Annual productions of dates, during the last six years (FAO Stat, 2004)

	Dates production in metric tons (Mt)					
Country	1999	2000	2001	2002	2003	2004
Africa	1, 920, 837	2, 063, 658	2, 187, 137	2, 148, 355	2, 153, 355	236,275
Algeria	427, 583	365, 616	437, 332	437, 000	437, 000	450,000
Benin	1, 000	1, 000	1, 000	1, 000	1, 000	1,000
Cameroon	320	340	360	380	380	390
Chad	18, 000	18, 000	18, 000	18, 000	18, 000	18,000
Djibouti	70	72	75	75	75	75
Egypt	905, 953	1, 006, 710	1, 113, 270	1, 115, 000	1, 115, 000	1,100,000
Kenya	1, 100	1, 000	1, 000	1, 000	1, 000	1,000
Libyan Arab Jamahiriya	114, 150	120, 000	140, 000	140, 000	140, 000	140,000
Mauritania	20, 000	22, 000	20, 000	24, 000	24, 000	24,000
Morocco	72, 561	74, 000	32, 400	33, 200	33, 200	54,110
Niger	7, 600	7, 600	7, 700	7, 700	7, 700	7,700
Somalia	9, 500	10, 000	11, 000	11, 000	11, 000	n.d.
Sudan	240, 000	332, 320	300, 000	250, 000	250, 000	330,000
Tunisia	103, 000	105, 000	105, 000	110, 000	115, 000	110,000
Asia	3, 669, 014	4, 081, 424	4, 247, 333	4, 220, 449	4, 226, 449	3,589,211
Bahrain	16, 774	16, 508	16, 508	16, 508	16, 508	17,000
China	115, 000	125, 000	117, 000	115, 000	120, 000	125,000
Islamic Republic of Iran	908, 340	869, 573	874, 986	875, 000	875, 000	880,000
Iraq	438, 000	600, 000	650, 000	650, 000	650, 000	n.d
Israel	10, 900	11, 732	9, 163	9, 200	9, 200	10,000
Jordan	1, 104	1, 320	1, 420	2, 110	2, 110	1,900
Kuwait	7, 894	10, 155	10, 376	10, 376	10, 376	10,500
Oman	282, 000	280, 030	298, 006	238, 611	238, 611	238,611
Pakistan	579, 880	612, 482	630, 281	650, 000	650, 000	650,000
Palestine	3, 852	3, 819	5, 051	5, 127	5, 127	5,500
Qatar	16, 389	16, 116	14, 230	16, 500	16, 500	16,500
Saudi Arabia	712, 000	735, 000	818, 000	829, 000	830, 000	830,000
Syrian Arab Republic	3, 000	3, 051	3, 921	1, 453	1, 453	1,500
Turkey	9, 400	9, 200	9, 200	9, 200	9, 200	9,400
United Arab Emirates	535, 964	757, 601	757, 601	760, 000	760, 000	760,000
Yemen	28, 517	29, 837	31, 590	32, 364	32, 364	33,300
Europe	7, 565	10, 717	11, 000	11, 000	11, 000	3,732
Spain	7, 565	10, 717	11, 000	11, 000	11, 000	3,732
Americas	23, 022	19, 949	22, 375	25, 374	21, 422	21,850
United States of America	20, 140	15, 785	17, 872	21, 954	18, 000	18,000
Mexico	2, 579	3, 965	4, 309	3, 172	3, 172	3,600
Peru	303	199	194	248	250	250
World	5, 620, 438	6, 175, 748	6, 467, 845	6, 405, 178	6, 412, 226	6,772,068

n.d.: not determined.

domesticated variety and had led to the current cultivars. The earliest cultivation of date palm had been recorded in 3700 B.C. (Munier 1973) in the area between the Euphrates and the Nile rivers. Then it was extended to other areas of the globe where the climate requirements of the plant are suitable, situated mainly between the parallel 9° and 39° North latitude (Munier 1973; Fig. 6.1) especially in dry and semi-arid regions. Hundreds of date palm cultivars are grown worldwide. Their fruits have different colours, flavours, sweetness, acidity and textures. The most popular and appreciated variety of dates in the world is mainly Majhool (originating from Morocco) and the most exported variety is Deglet Nour from Algeria and Tunisia.

P. dactilyfera belongs to *Arecaceae* (*Palmaceae*) family rich with over 200 genera and more than 2,500 species (Corner 1966) including *P. canariensis* (Canary island palm), *P. reclinata* (Senegal date palm) and *P. sylvestris* (Indian sugar date palm). The scientific name was derived from 'Phoenix', the legendary bird of old Greece, and 'dactylos' meaning 'finger' taking into account the shape of the fruit. Date palm is a dioeciously species where the male and female organs are carried by separate trees. It is the tallest tree among all the Phoenix species and the non-branching trunk can grow, under some conditions, higher than 30 m (Fig. 6.2). The plant has one terminal shoot apex that ensures the growth lengthwise. The root system of a date palm is highly developed. The leaves are large 4–5 m, long 4–8 m, alternates, pinnate, ground upward in a spiral pattern on the trunk and sheathing in dense terminal rosettes or crown of 100–120 leaves. The ends of the leaf are needle sharp, which seems to be an adaptation to protect the growth tip from grazing animals. Each leaf has an auxiliary bud that may be vegetative, floral or intermediate (Bouguedoura et al. 1990; Bouguedoura 1991). Auxiliary buds can form shoots commonly called offshoots or suckers during the juvenile life of the date palm and can carry inflorescences to maturity. The fruiting apparel emerges from auxiliary buds as clusters at the top of the tree among the terminal rosettes. Male and female flowers are issued on separate trees taking into account the dioeciously character of the species. Flowers are small and white on a richly branched spadix surrounded by a solitary, large spathe. The calyxes are cup-shaped, three-toothed while the petals are three-toothed, twice longer than the calyx in female flowers. The ovaries are three in general, but only one can develop into a fruit. The stamens are six with linear dorsifixed anthers. Pollination is generally wind-borne and artificial pollination of pistillate trees by placing cut portions of the male flower spikes on the receptive female inflorescence is, nevertheless, usually practiced and recommended to ensure high productivity. A fully productive date palm tree can support up to 10 clusters, which can carry more than 100 kg of fruits. Single fruit date or 'T'mar' in Arabic is usually cylindrical, occasionally rounded or ovoid, a drupe single seeded of 2.5–7.5 cm long × 4 cm large with fleshy, sugary pericarp, yellowish to reddish brown (Tackholm and Drar 1973; Purseglove 1972).

Production of dates around the world, according to FAO statistics, had peaked in 2002–2003 at 6,405,178 and 6,412,226 metric tons (Mt), respectively (Table 6.2). The world's largest producer over the past two years is Egypt with 1,115,000 mt followed by Islamic Republic of Iran (875,000 mt), Saudi Arabia (830,000 mt), United

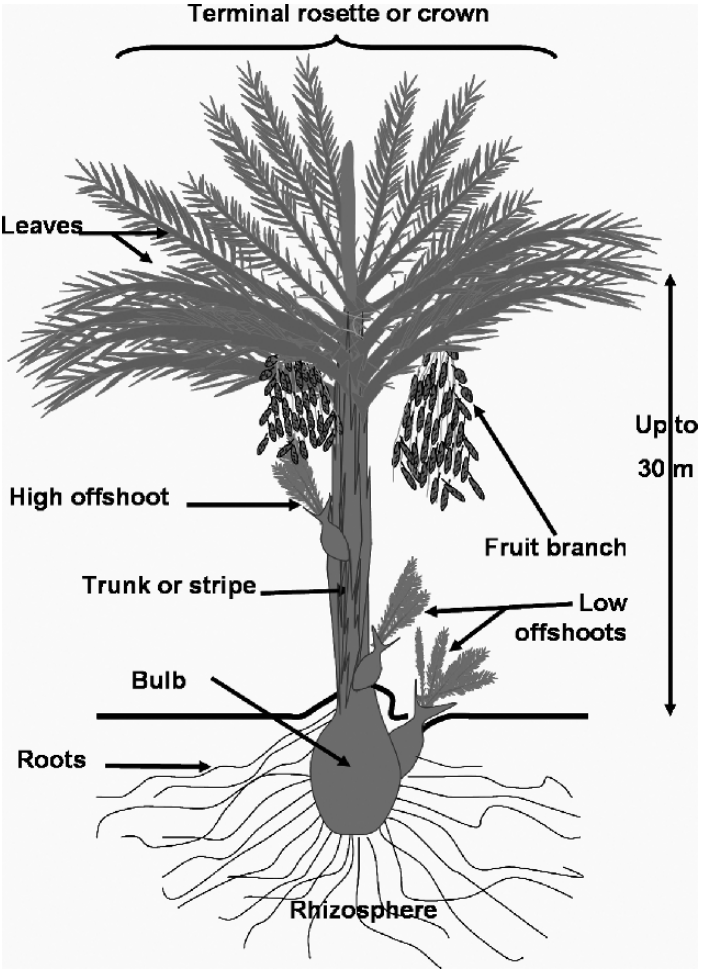


Fig. 6.2 Diagram representing the vegetative apparel of date palm

Arab Emirates (760,000 mt), Pakistan and Iraq (650,000 mt), Algeria (437,000 mt), Sudan and Oman (around 240,000 mt). Other significant producing countries are Libya, China, Tunisia, Morocco, Yemen, Mauritania, USA, Bahrain, Qatar, Spain and Kuwait.

The species is commonly propagated by offshoots and by seeds. Offshoots have a slow development and a healthy selected tree can produce from 0 to 3 offshoots per year and, in general, not more than 10–40 during its lifetime depending on the cultivar and the environmental conditions. Date palm normally begins to bear fruit within an average of 5–8 years after planting the offshoots; they reach their maturity at around 30 years and their decline begins after over 100 years of cultivation. Seed germination is the easiest but seedlings may take up to 10 years before flowering and fruiting occur.

Table 6.3 Comparison of propagation techniques

	Advantages	Disadvantages
Seeds	Easy and quick propagation Useful under-groves conditions Useful for breeding purposes Economic for selecting clones	Space, time and resources-consuming Produces heterogeneous population (a) Sex and pollen quality uncertain (b) Female plants attain maturity late Poor quality fruits
Offshoots	Produce individual 'true to mother' Bear fruits 2–3 years early	Space, time and resources-consuming Limited number of offshoots (c)
In vitro	Rapid propagation No seasonal effect on the plants Genetical uniformity ensured Exchange without risk of diseases and pests spread Economically reliable when large production is required.	Success in induction and maintaining callus nominal

The progeny derived from seeds are heterozygote and do not carry the same mother characters. Nevertheless, such propriety of sexual propagation is intended to create new genotypes and provides a basis for the selection of elite trees. In vitro propagation from tissue cultures is an alternative method for the mass propagation of date palm even though many difficulties have to be encountered in order to achieve that objective (Table 6.3). One of the limits in the mass production of plants through tissue culture techniques is undesirable plant off-types of a poor quality that can cause severe losses in the production and consequently affect the attributes of the whole process (Karp 1993; Cassells et al. 1999). Off-type production in plant tissue culture can result from stressful processes (Phillips et al. 1994; Skirvin et al. 1994) and can be searched in some cases for inducing genetic variability.

A seedling population shall have either of three attributes: (a) heterogeneous population: Mixture of individuals, often with a low production potential, poor fruit quality and late harvesting time (b) Sex, fruit and pollen quality of progeny cannot be determined before the first flowering about seven years after planting due to dioecious nature of the species (c) Offshoots are mainly produced in a limited number (20–30 per palm) during the early life of the plant (10–15 years from the planting) depending on the variety and on prior addition of fertiliser, irrigation and earthing around the trunks.

P. dactylifera is a widely distributed species covering an extensive geographic, soil and climatic areas. Almost 100 million date palm trees exist in the world with the vast majority located in the Middle East, North Africa and to a small extent in California and Mexico. The common requirement between all the date palm growing areas is the high temperature (35 °C, the optimum temperature for pollen germination) and the low relative humidity of the air necessary for fruit setting and ripening. Such desert tree requires large quantities of water ('even if its head is in fire its foot is still in water!'). Date palm grows in nearly rainless regions between 9 and 39° North Latitude, which is represented by the Sahara and Southern fringe of

the Near East (Arabia Peninsula, South of Iraq, Jordan). Both wild and domesticated cohabiting trees are morphologically and ecologically similar. Wild dates are of a small shape and non-edible compared to those coming from domesticated trees. Cross hybridisation among the two types of trees may still present in some regions, making the distinction of both types quite difficult.

Date palm is a high salt-tolerant tree and may harbour a good production even under 3,000 ppm of salty water. Some varieties had exhibited a high tolerance of total dissolved salt (22,000 ppm) but their productivity had been affected (unpublished data).

6.1.2 Socio-Economic Importance

The importance of date palm becomes, first, not only from its history as an inherited species for which cultivation was practiced by the oldest human civilizations, but also from its large ecological amplitude (highly adapted to arid conditions where the annual precipitation rarely exceeds 250 mm with a strong summer heat of about '50 °C' and cold winters of about '−10 °C'). Moreover, over 183,000 tons of dates with a trade value of about US $190,000 are marketed each year (Greiner 1996; Greiner 1998). Currently, date palm production bestows jobs for a population estimated at 50 million people. Thirty five percent of this manpower is localized in the

Table 6.4 Chemical constituents of dates

	Quantities per 100 g of dates	
Nutrients	Source 1	Source 2
Carbohydrates (g)	75.8	71.30
Fat (g)	0.4	0.45
Proteins (g)	2.5	1.97
Fibre (g)	3.9	n.d.
Ash (g)	2.1	1.58
H_2O (g)	15.3	22.5
Sodium (mg)	37.0	3.0
Potassium (mg)	680.0	652.0
Calcium (mg)	120.0	32.0
Phosphorus (mg)	50.0	40.0
Iron (mg)	7.3	1.15
Vitamins		
equivalentβ-carotene (μg)	26.0	50.0(UI Vitamin A)
thiamine (mg)	0.01	0.09
riboflavin (mg)	0.02	0.10
niacin (mg)	0.9	2.20
ascorbic acid (mg)	3.0	n.d.
Energy (Cal)	317.0	297.13

[1]Council of Scientific and Industrial Research, C.S.I.R. 1948–1976. The wealth of India. 11 vols. New Delhi. Fat (8% lauric, 4% myristic, 25% palmitic, 10% stearic, 45% oleic and 10% linoleic. Trace of capric and caprylic acids were found as well).
[2]USDA Handbook 8. n.d.: not determined.

Table 6.5 Organic and inorganic constituents among different cultivars

	Date Palm cultivars								
	Medjoul	Hayani	Deglet Nour	Zahidi	Amari	Halawi	Deri	Hadrawi	Barhi
Carbohydrates (g)	64	34	64	64	64	64	64	64	34
Fat (g)	0.4	0.1	0.4	0.4	0.4	0.4	0.4	0.4	0.1
Proteins (g)	2	1	2	2	2	2	2	2	1
Fibre (g)	6	5	6	6	6	6	6	6	5
Sodium (mg)	3	2	3	3	3	3	3	3	2
Potassium (mg)	650	320	650	650	650	650	650	650	320
Magnesium (mg)	35	15	35	35	35	35	35	35	15
Iron (mg)	2	2	2	2	2	2	2	2	2
Energy (Cal)	268	140	268	268	268	268	268	268	140

Source: http://www.hadiklaim.com/dates_values.asp

southern Mediterranean countries (Ferry 1996). In the majority of these countries, particularly Morocco, date palm creates a micro-climate in the vast desert allowing the settlement of several subjacent cultures, which constitutes the principal subsistence cropping for a considerable human population and their animals. Dates are very rich in carbohydrate, minerals and vitamins (Tables 6.4, 6.5), which can give the necessary metabolites to humans far away from the luxury of stores (Duke 1983). Dates are low in fat and high in carbohydrates, fibres, potassium and vitamins (Tables 6.4, 6.5). They can be dried on stalks or spread out on mats. Date products include syrup, "dibs" (an indigenous honey made from the juice), jam, chutney, vinegar and fermented date juice.

The date groves provide shade for a variety of other crops, such as cotton, maize, citrus fruits, pomegranates, alfalfa, vegetables, mango and cereals. Various trees are also often found between the date palm trees. The leaves are often dried, dyed and then plaited into mats, hats, trays or baskets; they can also be used to attach various materials and to make cords; the wooden midribs are used for roofing. In the past, the midribs were also used to make the dome-shaped fishing cages, now made of metal. The palm trunks provide a sturdy building material or used in the construction of fishing boats in some countries.

6.2 Constraints and Challenges

6.2.1 Constraints

Date palm culture face many constraints that are mainly due to its development under hostile desert conditions, which requires high tolerance to drought and salt stresses and a growth without regular amendment supply. Adaptation of different date palm genotypes to such conditions and variation in yield might occur. Date palm also face many biotic constraints especially *bayoud* caused by *Fusarium oxysporum* f. sp. *albedinis* (Malençon 1934; Carpenter and Klotz 1966; Louvet and

Toutain 1973; Laville 1973; Djerbi 1988). This vascular fusariosis is the most devastating disease of the date palm trees. It has been described, first, in the south of Moroccan groves. Currently, it is still spreading through North African countries especially in Morocco and Algeria where more than 12 million date palm trees have been destroyed so far. No efficient mean is known to control this disease and only few cultivars with a poor quality of dates, unfortunately, are known to be resistant to bayoud (El Hadrami et al. 1998). Recently, a new date palm disease called 'maladie des feuilles cassantes' or brittle leaf disease was recently discovered in Tunisia (Triki et al. 2003), where more than 40,000 trees were destroyed within a short period of time. However, the cause of such wilting is still undetermined. Another constraint that appears on top of the list for date palm is the fact that current trees in different oases' groves are getting old and their replacement through natural offshoots propagation is not sufficient to maintain such perennial crop. The lack of national programmes aimed at the promotion of date palm culture in the rural communities increases the impact of this problem and leads the farmer to change this culture to another to ensure their agricultural incomes.

6.2.2 Breeding Challenges

Taking into account the date palm constraints many challenges are directed towards the breeding programme. These challenges vary between countries and can be classified into three levels as short-, mid- and long-term challenges depending on the objectives. The short-term challenge is to maintain the genetic diversity within groves and to reduce the use of monovarietal cultures, which leads to the impoverishment of the genetic pool. Another short-term objective is to replace and provide oases' groves with juvenile material in order to ensure the perennial aspect of the date palm culture. The mid-term challenge represents the core of the breeding programme wherein resistances to different biotic and abiotic stresses are pursued and the 'date palm complex system' is studied. As for the long-term objective, the creation of new cultivars using conventional and/or non-conventional approaches would lead hopefully to resolve some of the date palm constraints especially to "eradicate" or at least reduce the impact of the bayoud disease.

Facing these challenges, one can notice that only a little has been done with regard to the breeding programme of this species. Several attempts have been conducted in the past but they have led to only a few achievements due to the difficulty encountered such as the slow plant growth and its dioecious character.

6.2.2.1 Genetic Resources and Yield Variation

The genetic pool in terms of date palm varieties is rich but not well known and characterised depending on the country (about 5,000 varieties claimed). Most of these varieties are not grown any more in the groves because high productive and homogeneous individuals were preferred to them. Many concerns have been raised

against the impoverishment that the date palm groves are encountering since the last three decades.

The establishment of new and industrial plantations of date palm around the world will certainly lead to an impoverishment of the genetic pool. These plantations have been established taking into account the dates trade outcomes and the adaptation of some varieties to several conditions depending on the country. In Tunisia for instance, the cultivation of the best trade-marketable producing-date variety Deglet Nour may represent more than 65% of the plantations. Similar trend of plantation of the variety are reported in Algeria. In the south of Morocco, Boufeggous and Jihel are the two varieties most appreciated in the Valley of Draa, while Mejhoul is the best variety adapted to the conditions of Tafilalet region. These practices of monovarietal cultures are very treacherous because they could lead within a short period of time to a high reduction of the date palm biodiversity.

6.2.2.2 Breeding Objectives

Date palm breeding is mostly based on conventional methods. Advances in selection for important agronomical traits, such as fruit quality or yield, and disease resistance are difficult due to the time-consuming generation of new individuals of the species. Moreover, the identification of trees is not usually possible before the onset of fruiting, which takes 3–5 years after the planting. Varieties description is mainly based on morphological markers, such as those of the fruit, which are complex and greatly submitted to the effect of the environment (Sedra et al. 1998). During the last two decades, biochemical markers such isozymes and proteins have developed to ensure an effective genotypic identification (Bendiab et al. 1993; Bennaceur et al. 1991; Fakir et al. 1992). However, these markers encounter many limits due to their sensitivity and willingness of detecting genomic variation. Recently, amplified fragment length polymorphism (AFLP) have been shown to be advantageous markers in terms of studies of the date palm genetic diversity, identification of genotypes (Al Khalifah and Askari 2003) and diagnostic of the pollinisation patterns among and within groves. The same markers are also used during the process of the clone's micro-propagation in vitro in order to differentiate between genotypes and chase the genetic stability of their derived clones. For this purpose, the uses of molecular marker has become an advantage as compared to the traditional way based on morphometry and/or cytogenesis approaches. On the other hand, genetic engineering and molecular markers (Zietkiewicz et al. 1994; Zehdi et al. 2004), although they are of utter significance, have not been yet successfully used in date palm breeding due to long period required for regenerating the crop.

Several investigations aimed at the study of bayoud resistance characteristics have been carried out by many authors (Sedra 1995; El Hadrami et al. 1997; El Hassni et al. 2004) as one of the major component of the IPM strategy that aims to overcome this disease. Several breeding programmes of date palm have been instigated in Morocco (Louvet and Toutain 1973, Saaidi 1979, 1992) and Algeria (Fernandez et al. 1998). Their objectives were to identify date palm genotypes among the Moroccan and Algerian groves with a high or a suitable resistance level

to bayoud and high quality of dates that might constitute parents for the breeding programmes. After selecting the wild genotypes with the suitable characteristics of either resistance or fruits quality, male flower spikes were collected from trees showing high degree of resistance to bayoud or an effect on the fruit set and placed on the receptive female inflorescence known to be of a high quality of fruits or resistant to bayoud, respectively. These cross hybridisations have led to generate new genotypes that have been introduced under greenhouse and in vitro for multi-annual tests for the resistance after field checking of the quality of the fruits. Other attempts at production of new genotypes with resistance to bayoud have been conducted in USA, Algeria and Morocco, but so far did not lead to the expected results (Bouguedoura 1991) due to the difficulties encountered in working with date palm, requiring a long time for regenerating F_1 and F_2 progenies and for back-crossing them (up to 30 years approximately). It is therefore straightforward understandable why only a little has been achieved in the conventional date palm breeding programme.

Similarly to the breeding for resistance to the bayoud, and to the best of our knowledge, nothing has been described concerning the breeding for other abiotic constraints such as drought and salinity mainly due to the same difficulties.

Tremendous advances have been made with regards to the study of date palm genetics. The determination of the number of chromosome has been investigated by many authors who have reported different chromosome numbers. In 1910, Nemec had reported for this diploid species $2n = 28$ while he was working with young developing embryos of a non-specified cultivar. Later, other authors have stated that the chromosomes number is $2n$=32 or 36 (Beal 1937; Al Salih and Al Rawi 1987; Al Salih et al. 1987; Al-Salih and Al-Jarrah 1987; Ibrahim et al. 1998) or 26 (Loutfi 1999). These differences in the evaluation of chromosome numbers are most likely due to difficulties in obtaining soft tissues in mitosis especially from adult trees where the chromosomes are small and numerous. The development of a cytological method based on chromocyanin staining (Siljak-Yakovlev et al. 1996) has also shown the occurrence of sexual chromosomes carrying distinctive nucleolar heterochromatin. On the other hand, studying the genomic size of date palm using flux cytometry and Arabidopsis as a standard, Ouenzar et al. (2001) had estimated the 2X DNA to 490 Mbp (0.51 pg). Comparing this result to other findings on monocots and perennial plants, it seems that date palm has a small genome.

So much needs to be known about date palm in terms of molecular genetics in order to better understand the genetic diversity of the species. Up-to-date, no hands-on molecular method is available for distinguishing date palm female producing trees from males before the first flowering about 5 years after planting. Also, it is difficult to identify female clones according to their morphological characteristics other than at the fruiting time.

At present, about 5,000 cultivars have been identified for date palm within the 34 producing countries. They are fundamentally distinguished on the basis of their fruit characteristics, which are heterogeneous and environmentally dependant. It is, hence, necessary to develop efficient systems based on neutral molecular markers that might be useful in determining the sex within progenies as well as the traits controlling fruits quality and resistance/tolerance genes to bayoud and other biotic

and abiotic stresses. Many efforts have been directed towards achieving this objective and numerous isoenzymatic systems have been tentatively used to characterise date palm cultivars (Torres and Tisserat 1980; Stegemann et al. 1987; Baaziz and Saaidi 1988, Chandra-sekhar and de Mason 1988; Bennaceur et al. 1991). Many of these studies have used either fruits (Stegemann et al. 1987), seeds (Chandra-sekhar and de Mason 1988) or leaves belonging to seedlings of known parents (Torres and Tisserat 1980, Bendiab et al. 1993) or adult plants (Baaziz and Saaidi 1988; Bennaceur et al. 1991). More recently, molecular markers such as RFLP, RAPD, mitochondrial minicircular plasmid-like DNAs and AFLP have been developed in order to identify and/or characterise date palm cultivar of either known or unknown genotypes or to establish phylogenetic relationships between genotypes carrying fruits of a good quality (Ait-Chitt et al. 1993; Benslimane et al. 1994; Cornicquel and Mercier 1997; Bouachrine 1997; Sedra et al. 1998; Ben Abdallah et al. 2000; Trifi et al. 2000; Trifi 2001; Al Khalifah and Askari 2003). On the other hand, research on the biochemical dissection of the mechanism of resistance to bayoud or to other abiotic stresses are still ongoing and several biomarkers of phenolic nature or different matters of antifungal compounds have been identified (Ziouti et al. 1996; El Hadrami et al. 1997; Ramos et al. 1997).

6.3 Progress in Biotechnologies

Both conventional and non-conventional breeding programmes of date palm rely at certain time points on biotechnologies and specifically the use of tissue culture for transformation and/or regeneration of plants. Many advances have been made in this field of investigation regarding the determination of condition factors and stimuli that control the date palm tissue plasticity and totipotency. These two notions, as in many other systems, are still empirical making the identification of culture conditions and stimuli extremely difficult to gather. The breeding programmes will be more appreciated when the in vitro regeneration of date palm can be controlled.

6.3.1 Date Palm Tissue Culture

Date palm is among the small number of crops where in vitro techniques including organogenesis and somatic embryogenesis have completely or partially replaced traditional vegetative propagation practices depending on the country. Many date palm tissues such as leaves, apical dome, shoot tips, lateral buds and roots have been proven to be useful as explants for initiating tissue culture (Zaid and Tisserat 1983). Their plasticity, which allow them to change their metabolism, growth and development to best suit a specific environment controlled by specific stimuli, subsequently leads to the regeneration of whole plants. However, with this monocotyledon species, many attempts of tissue culturing were facing a browning (El Bellaj

and El Hadrami 2004; El Hadrami 1995) followed by the rapid death of the tissues depending on the cultivar.

Similar to other plant systems, initiating in vitro tissue cultures of date palm requires to meet both chemical and physical needs by providing the culture vessel with adequate growth medium and external environment (quality and duration of light, temperature, pH, gaseous environment, osmotic pressure, etc.). In the date palm case, adding some antioxidants into the MS medium (Murashige and Skoog 1962) is critical because of the ability of the tissues to rapidly get oxidized. Activated charcoal has been reported in many studies as an agent that minimise the effect of tissue browning under induction conditions for embryogenic cultures (Tisserat 1979; Sharma et al. 1984; Bhaskaran and Smith 1992; El Hadrami 1995; Loutfi 1999). Other antioxidants such as polyvinylpyrrolidon (PVP) and ascorbic acid have also been used (Poulain et al. 1979; Beauchesne et al. 1986). Also, depending on the type of culture, carbon sources other than sucrose might be worth the use to initiate or propagate date palm tissue cultures (Zouine and El Hadrami 2004).

Culture media used for the in vitro cultivation of date palm require the use of the macroelements and the micro-elements described by several authors (Tisserat 1979; El Hadrami 1995, 1998; El Bellaj 2000; Fki 2005). Other organic supplements such as thiamine and carbon source might need to be added in a specific form to achieve the process of generating plantlets.

Plant tissue culture, including that of date palm, requires a balance of plant growth regulators during their different steps in vitro. The type, amount and ratios of the growth regulators are a critical point in generating plantelets from date palm tissue culture. Depending on the step of induction, multiplication or organogenesis,growth regulators ratio such as the ratio of auxin to cytokinin are to be adjusted. Intermediate ratios of auxin to cytokinin are usually used to initiate formation of calli from date palm while low ratios are preferably used during the shoot regeneration step.

6.3.2 In Vitro Culture Methods of Date Palm

Tissue culture such as callus, cell-suspension cultures, root cultures, shoot tip and meristem, embryo or micro-spore cultures have been developed for date palm with more or less success in generating plantlets (El Bellaj 2000; Fki 2005; Fki et al. 2003).

6.3.2.1 Organogenesis

The regeneration of date palm plantlets through organogenesis involves the achievement of several steps that are more or less critical: (i) meristem induction; (ii) shoot multiplication; (iii) shoot elongation; and (iv) acclimatisation.

Organogenic cultures are usually induced from the internal basic side of young leaves carried by the offshoots. Induction generally requires no light in order to minimise the accumulation of phenolics and tissue browning as well as the stimu-

lation of cell division. This step takes four to six months under the aforementioned normal conditions. It is mediated by the interaction of several factors including the composition of the culture medium, the genotype and the time taken between the offshoot collection from the mother plant and its introduction in vitro.

The induction medium consists, in general, of regular MS medium amended with one of the following combinations of plant growth regulators that provide optimum conditions for induction of organogenic cultures: (i) 5.4 μM NAA (1-naphthaleneacetamide), 4.9 μM IBA (indolebutyric acid), 5–27 μM NOA (2-naphthalenyloxyacetic acid) and (ii) 0.5 μM 2iP (*N*-(3-methyl-2-butenyl)-1*H*-purin-6-amine) or 5.4 μM NAA, 5.7 μM IAA (indol-3-ylacetic acid), 5–27 μM NOA, 0.5–14.8 μM 2iP (Poulain et al. 1979). Even under the same culture conditions, there might still be significant variation observed in terms of frequency of induction among cultivars. On the extreme end, each date palm genotype might require a specific culture medium as shown by several authors. In addition, explants from offshoots and inflorescences can develop roots much earlier than form shoots leading to an inhibition of a further caulogenesis (Loutfi 1999). Meanwhile, groups of cultivars have been distinguished to have the same behaviour in terms of percentage of shoot and callus formation (Loutfi and Chlyah 1998).

The multiplication of shoots normally requires plant proliferation medium in which the auxin to cytokinin ratio is higher than 1. Upon such a proliferation medium, the multiplied shoots bear a resemblance to rosettes. Physiological disorders such as hyperhydricity are often observed in these cultures, but factors affecting their regulation have not yet been clearly identified. Preliminary studies have indicated that the high levels of ammonium nitrate could enhance the rapid growth and the hyperhydricity of date palm cultures.

Elongation of offshoots is frequently obtained after a transfer of the shoot buds into a growth medium with a high auxin to cytokinin ratio (Beauchesne et al. 1986; Loutfi and Chlyah 1998). Approximately 1–2 years after induction, date palm plantlets can be regenerated and transferred to the greenhouse for evaluation/screening. Roots formation occur easily during the final stages of in vitro culture.

6.3.2.2 Somatic Embryogenesis

Embryo-like structures of date palm derived from somatic (asexual) embryogenesis are able to develop into a whole plant in a similar way as zygotic embryos. Till date, two consecutive steps have been known to generate date palm plantlets through somatic embyogenesis (Poulain et al. 1979; Reynolds and Murashige 1979; Tisserat 1979). Poulain et al. (1979) have firstly described the initiation of vegetative buds from date palm offshoots heart. Later on during the same year, Reynolds and Murashige (1979) then Tisserat (1979) have described the induction and regeneration of somatic embryos from these cultures and utilized other various tissues as explants. Tisserat (1979) used plant growth medium supplemented with activated charcoal and 450.5 μM of 2,4-D (2,4-dichlorophenoxyacetic acid) that is higher than the most commonly used concentrations. This technique is being successfully

used for large-scale micro-propagation of date palm by somatic embryogenesis in numerous commercial laboratories. More recently, little improvements have been reported regarding the use of somatic embryogenesis in date palm system (Zaid and Tisserat 1983; Daguin and Letouzé 1988; Bhaskaran and Smith 1992; El Hadrami 1995; Sharma et al. 1996; Loutfi 1999; El Bellaj 2000; Zouine and El Hadrami 2004; Al-Khayri 2005).

Critical factors controlling the establishment of date palm embryogenic cultures include explant type, genotype and plant growth regulators. Either offshoots or flower buds were successfully used as explants to regenerate embryogenic cultures. Optimal results were obtained using 2,4-D (2,4-dichlorophenoxy-acetic acid) as growth regulator even though other auxins such as picloram, 2,4,5-T (2,4,5-Trichlorophenoxyacetic acid), NAA naphthaleneacetic acid, IAA (indolacetic acid) and NOAA (naphthoxyphenoxyacetic acid) have led to more or less inducible embryogenic callus cultures. Early studies had suggested that the use of higher concentrations of 2,4-D (450.5–901 μM) in the presence of activated charcoal are necessary for the induction of embryogenic cultures, while more recently it has been demonstrated that lower concentrations of 2,4-D are enough to achieve that goal (El Hadrami 1995; El Hadrami and Baaziz 1995; El Hadrami et al. 1995). The induction medium consists of a semi-solid modified MS containing de Fossard vitamins (deFrossard 1976), 22.6 μM 2,4-D, 22.2 μM benzyladenine (BA) and 150 $mg.l^{-1}$ of activated charcoal (El Hadrami 1995; El Bellaj 2000; Zouine et al. 2005). The explants are placed in the dark for 4 months under $26 \pm 2\,^{\circ}C$ and sub-cultured on the same medium every 4–5 weeks. On the other hand, differences were observed among date palm genotypes in terms of embryogenic potential (El Hadrami 1995; Loutfi 1999; El Bellaj 2000), which is ascribed to the biochemical and histological changes occurring in the cultures (El Hadrami 1995; El Hadrami and Baaziz 1995; Baaziz et al. 1994). Those changes include a higher variation in terms of phenolics contents, proteins and peroxidases activities. Date palm embryogenic cultures are generally characterised by an accumulation of flavonoids that would indicate an acquisition of an embryogenic potential. Meanwhile, an increase of the activity of some isoforms of peroxidases can be also observed (El Hadrami 1995; El Hadrami and Baaziz 1995; Baaziz et al. 1994; Fki 2005) leading to many tissue browning effect. In many cases, tissue browning for embryogenic cultures can be avoided by adding activated charcoal or other antioxidants such as PVP and ascorbic acid (Tisserat 1979; Sharma et al. 1984; Bhaskaran and Smith 1992; El Hadrami et al. 1995; Loutfi 1999; Poulain et al. 1979; Beauchesne et al. 1986).

Maintaining embryogenic cultures can be achieved on the same semi-solid modified MS medium used for the induction and containing de Fossard vitamins (deFrossard 1976), 22.6 μM 2,4-D, 22.2 μM BA, and 150 $mg.l^{-1}$ of activated charcoal (El Hadrami 1995; El Bellaj 2000; Zouine et al. 2005). Other culture mediums also allow a rapid proliferation of embryogenic-suspension cultures (Sharma et al. 1986; Daguin and Letouzé 1988; Bhaskaran and Smith 1992; El Bellaj 2000; Fki 2005). The growth medium for suspension cultures consists of MS medium containing 0.45 μM BAP (Benzylaminopurine), 2.22 μM, 2,4-D, 100 $mg.l^{-1}$ glutamine and 10^{-7} M abscissic acid. About 0.5–1 g FW transferred into 50 ml Erlenmeyer

flask containing 20–25 ml of medium are enough to induce a new embryogenic-suspension cultures. Suspension cultures are maintained at 100 rpm in the dark at 26 °C. The suspensions are sub-cultured each 15 days upon the same medium. Other protocols have been recently described concerning the establishment of the embryogenic suspension cultures in date palm (Fki 2005).

Somatic embryos development involves the use of modified semi-solid MS medium supplemented either with 2.3 μM 2,4-D and 0.44 μM BA or without any phytohormones (El Bellaj 2000; Zouine and El Hadrami 2004; Fki 2005).

Mature somatic embryos can germinate on medium without plant growth regulators. However, the percentages of recovery obtained are lower and does not meet the expectations and much progress in this area of research are still ongoing (Zouine et al. 2005; Fki 2005).

Both organogenesis and somatic embryogenesis are being used to produce large numbers of date palm plants on a commercial scale. Each technique has certain limiting factors while having its particular advantages. The main limits of plant regeneration from both pathways are the tissue browning, the late response of explants and endophytic contaminations. Callus and morphogenic cultures of date palm have been induced from different explants, including zygotic embryos, roots (Eewens 1978; Sharma et al. 1980), young leaves (Sharma et al. 1984), shoot tips (Zaid and Tisserat 1983; Gabr and Tisserat 1985), fragments of stems excised from seedlings, bases of young leaves obtained from the hearts of offshoots (Beauchesne et al. 1986; El Hadrami 1995), fragments of young inflorescences (Drira and Benbadis 1985; Bhaskaran and Smith 1992; Loutfi and Chlyah 1998) and indeterminate auxiliary buds (Bouguedoura et al. 1990). The most commonly used explants consists of segments taken from the hearts of offshoots and that often contain auxiliary buds (Poulain et al. 1979; Beauchesne et al. 1986, Sharma et al. 1986; El Hadrami et al. 1995; Veramendi and Navarro 1996). Floral segments have also been frequently used as well (Drira and Benbadis 1985; Bhaskaran and Smith 1992; Loutfi and Chlyah 1998; Loutfi 1999; Fki 2005). Endophytic microbial contamination is a major problem and consists primarily of *Bacillus* spp. Antibiotics such as Gentamycin were tentatively used by some authors with uneven success (Cherkaoui 1997).

6.3.2.3 Haploid Recovery from Anthers or Ovules

Studies dedicated to anther and ovule cultures recovery are scarce for date palm. Attempts under various conditions have led to cell divisions and to the formation of globular embryoids from uninucleate micro-spores. For some of the successive attempts, cold treatment combined to the use of two auxins and one cytokinin have been proven to be the key elements to generate embryoids (Bouguedoura 1991; Chaibi et al. 2002) that unfortunately were unable to develop. Investigation of different treatments and various exogenous factors had remained without any significant positive effect unless there was a formation of the weak calli surviving only during a short period of time. The main difficulties encountered in such studies are related to the short-time flowering period that does not allow usually having enough

fresh anthers with uninucleate micro-spores. Furthermore, date palm male anthers typically turned brown and died a few weeks after their culture. Chaibi et al. (2002) reported the definition of the most suitable stage for anthers to be treated with a thermal choc treatment at 37–38 °C prior to their in vitro culture. These authors reported also the use of MS medium amended with 2,4-D, 2-isopentenylaminopurin (2-iP) and activated charcoal to prevent the tissue browning, which had allowed them to observe an increase in the percentage of micro-spore division.

Some haploid recovery attempts have concerned also date palm unfertilised ovules. Due to the small size of these ovules, browning and necrosis were the main limits encountered by these cultures. Although the carpels enlarged and became quite prominent when cultured, the use of activated charcoal is required to ensure them a much longer survival and roots or callus formations (Bouguedoura 1991). Up-to-date, the best results ever obtained were from flowers taken from closed spaths and in which the embryo sacs were formed that contained undifferentiated cells.

6.3.2.4 Genetic Manipulations of Protoplasts or Cell Suspension Cultures

Till the time of writing this chapter, no study or report have been published regarding date palm protoplast isolation and culture even as several attempts were carried out in many laboratories around the world (Algeria, Tunisia, USA). Serious difficulties were encountered to overcome the browning and rapid death of protoplasts. Few reports describe the use of cell suspensions as a tool of genetic manipulation of date palm. It has also been shown that embryogenic cells could be irradiated and submitted to toxins coming from *Fusarium oxysporum* f.sp. *albedinis*, the agent causing the Bayoud, to screen resistant individuals (Fki 2005).

Date palm as a fruit tree could be irradiated in vitro as micro-cultures (Ahloowalia and Maluszynski 2001). Induced mutation in this system would be an effective way of introducing variability within the wild and the bred stock as reported for other crops (Maluszynski et al. 1995; Szarejko et al. 1995; Jain 2006). This approach can be used in conjunction with the regeneration of the material either through somatic embryogenesis or organogenesis. Somatic embryos often start off a single cell, which makes them an ultimate candidate for applying induced mutagenesis with less chimerism (Jain 2002; Ahloowalia 1997). However, somatic embryos germinate at a very poor rate that make them worthless for a large-scale multiplication based on mutation induction (Jain 2002).

Induced mutagenesis use either ionizing radiation such as X- or γ-rays and neutrons or chemical mutagens for inducing variation (Ahloowalia and Maluszynski 2001). Combined to various in vitro cultures induced mutagenesis represents the simplest, fastest and highly efficient method for improving crops. It can result in the development of mutant cultivars able to exhibit resistances to biotic or abiotic stresses, to produce a desirable quality and/or an improved quantity of fruits as well as specific morphological features (Jain 2002). Moreover, in vitro techniques nowadays allow the induction of mutations in a large number of propagules within a reduced working space. Several cycles of sub-culture carried out over a short period

of time can be sufficient to screen for the true and stable mutations. Many breeding programmes around the world use this technology to induce variation within the stock. Maluszynski et al. (1992) and Ahloowalia and Maluszynski (2003) reported that more than 1,800 cultivars out of the collection maintained by the FAO/IAEA division of the Nuclear Techniques in Agricultures are/were either direct mutants or derived from crosses involving individuals subjected to induced mutagenesis, and those cultivars have been released in more than 50 countries. The improvement of in vitro techniques for date palm has made it possible to irradiate these cultures in a large scale and to maintain them within the same collection (Jain 2006). Moreover, recombinant DNA research and the use of the model species *Arabidopsis thaliana* have been active during the last two decades in providing labeled probes such as RFLP, micro-satellite based DNA fingerprinting, developed for cloning and mapping plant genes or transgenesis that are able to trace such modifications within the genome. Nowadays, it is routine to identify and analyse mutants using DNA fingerprinting or mapping genetic alterations using PCR based markers, such as RAPD, AFLP, SSR, SNP... and thus tagging mutants (Beetham et al. 1999; Zhu et al. 1999). Stable mutations can be then linked to noticeable changes in the DNA sequence of specific plant traits, then mapped and located on the chromosomes before being analysed by functional genomics and transferred into a desirable background variety. In turn, breeding processes can be accelerated to lead to new varieties of crops enhanced for either their yield or quality or resistance to biotic or abiotic stresses.

6.3.2.5 Cryopreservation

The first attempts at freeze preservation of date palm go back to the late 1970s when Finkle et al. (1979) and Ulrich et al. (1979) investigated this possibility. Later on in the mid-1980s, Tisserat et al. (1985) reported the cryopreservation of pollen dusted on freshly opened spathes of 10-year-old Deglet Noor palm tree. The freezing of pollen had no effect on the fruit yield and developments as compared to the non-frozen pollen.

Bangniol et al. (1992) suggested that for cryopreservation of date palm, gradients may be exhibited both for outflow of water and the penetration of the cryoprotectants. MyCock et al. (1997) has reported that late globular and early torpedo stage date palm embryos can continue their normal growth and development after cryopreservation. To do so, embryos have to be pre-treated with a cryoprotectant mixture of glycerol and sucrose, then dried to a water content of 0.4–0.7 g/g. Prior to that, Mater (1987) had reported that callus of date palm could be treated with a cryopreservation for 4 months at −25 °C within a mixture of PEG (polyethylene glycol), glucose and DMSO (dimethyl sulfoxide). The freezing under these conditions did not affect the potential of the calli to be embryogenic once they were unfrozen; however, their growth was slowed down during the first 2 months of culture. Some results on the same subject have been recently reported (Fki 2005) showing the feasability of this technique.

The main goal of date palm pathologist, breeders and agronomists is to construct a *germplasm* collection, to be able to retrieve it using easy and versatile tools and preserve it long-term without inducing any variability. Embryogenic date palm calli were subjected to cryogenic treatments and stored in liquid nitrogen at −196 °C for several months. In some cases, calli was invigorated after a quiescent period of 4–8 weeks and had regenerated plantlets (Tisserat 1982). To control the conformity of this technique, the polymorphism observed based on five enzymatic systems (alcohol dehydrogenase, esterase, peroxidases, phosphoglucomutase and phosphoglucoisomerase) was analysed in the leaves of the regenerated plantlets. Isozyme patterns observed for regenerated plants from frozen calli were similar to those regenerated from unfrozen calli.

Other studies had reported a protocol for pollen-handling as well as the characteristics for its hydration/dehydration in preparation for long-term storage (Kristina and Towill, 1993). More recently, a long-term method for preserving date palm tissue cultures was reported using in vitro shoot bud and callus cultures (Bekheet et al. 2002). After 12 months of incubation at +5 °C in the dark, a relatively high percent of cultures had remained viable. Other studies are ongoing to test more long-term cryogenic storage with different date palm tissues.

6.4 Conclusions

Tremendous progress has been made during the last few years regarding the in vitro regeneration of date palm from zygotic or somatic explants through either organogenesis or somatic embryogenesis. This progress has made it possible to afford large production of vitroplants even though the regeneration process is still to be shortened to meet the needs of the industry. Many of the physiological mechanisms controlling various stages of in vitro development of tissues remain non-elucidated and numerous studies are carried out in different laboratories to understand them better. Also, it is necessary to develop reliable molecular markers that are able to trace and discriminate genotypes as well as their sex within a progeny. The breakthrough made in terms of bioreactor technology development, a process by which somatic embryogenesis will reach the industrial level, seems very promising. In a such process, cotyledonary stage embryos grown under high photosynthetic photon flux (PPF) to allow them to become photoautotrophic or nodular callus could be transferred into different types of culture systems, such as Magenta vessel, RITA-bioreactor (modified system to improve air exchange), temporary (root zone) immersion bioreactor system (TRI-bioreactor) with forced ventilation, to achieve large-scale embryo-to-plantlet conversion. Even if there is still a need for speeding up these systems of culturing plant in vitro, the potential success of such techniques in multiplying a few crops such as coffee or strawberries (Hanhivena et al. 2005; Afreen et al. 2002) has opened a new era of a rapid and economical way of developing new cultivars massively and plan for a large commercialisation. One would hope to see these techniques applied to the multiplication of date palm in the near

future since many advantages are to be expected. Clonally-propagated plants can produce high quality and uniform seeds; improve the breeding process as well as the vigour and quality of the progenies. In addition, clonal propagation can produce disease-free *germplasm* proper for international exchange, decreasing thus the labor, production and testing expenses of hybrid seeds. One would besides wish for a settlement of a date palm breeding programme based on molecular maker assisted selection in the next few years. The most suitable markers, out of those discriminating the sex within a progeny, would be markers for the productivity of date palm trees (yield and quality of dates) and for the resistance/tolerance to bayoud and other biotic and abiotic constraints. Also, it is necessary to be aware of the danger from increasing productivity using monovarietal plantations. One should keep in mind that the sustainability of date palm groves relies on the preservation of the biodiversity of the crop within the groves. Management of date palm cropping should hence be planned in a monoculture composed of various cultivars rather than a perpetuation of hectares covered by monoline cultures.

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Chapter 7
Litchi Breeding for Genetic Improvement

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7.1 Introduction

Litchi chinensis Sonn. ranks (high) among the most important horticultural crops, belongs to the family Sapindaceae and widely grown in tropical and subtropical regions (Menzel 1985). The tree produces delicious top quality fruits that are in great demand for their wholesome taste, sweet aroma and attractive colour. They are mainly consumed as a fresh table fruit worldwide but in China, dried litchis, called litchi nuts with the taste of the raisin are quite popular. They are also preserved and canned in syrup or used as squash. In Florida, frozen fruits are consumed on a limited scale. China, India and Taiwan are the major producers of litchi whereas in the last 40 years substantial increase in production in South Africa, Australia, Thailand, Vietnam, USA and Israel has led litchis to become a significant commodity in the international trade, (Underhill et al. 1997). Major thrust of litchi research has been on prevention of physiological browning and retention of bright red colouration of litchi fruits in several countries.

Exotic litchi fruits have received worldwide attention. Increase in popularity has necessitated litchi cultivation in a wide range of environmental conditions. In fact litchi is generally adapted to various soil types via alluvial sands, loams, heavy clay, organic soil and calcareous soil with 30% lime and rock files (Chapman 1984a). In China, the best litchi trees are prevalent in Gwanagdong province close to the rivers on alluvial sands with good drainage and access to the water table (Chapman 1984b). They are also grown in gravelly sandy loam to loam soils as well as in swampy areas. However, soil in Fijian province is very high in clay, poorly drained and acidic in reaction (Winks et al. 1983). In South Africa, trees are more vigorous in growth on acid soils rather than on neutral or alkaline soils (Marloth 1947). Under Indian conditions, litchi cultivation in Bihar state is common on calcareous soils containing more than 40% free calcium carbonate and trees flourish well in a moist subtropical climate and in deep loamy soil with high moisture content. However,

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it has increasingly gained popularity in the Bihar plateau (Kumar 1977) and sub-mountain region of Punjab (Singh and Sarin 1957). A sandy loam or clay loam with pH 5.5–7.0 having considerable soil depth and minimum of 1.5–2 m down water table with non-stagnating water is considered optimum for the growth of litchi trees.

Litchi exhibits appropriate growth in a climate characteristic of the area of its origin. Frost in winter and dry intense heat in summer are detrimental to litchi cultivation although periodic cold snaps in winter between 1 and 5 °C appear to be essential for fruit bearing. However, total duration of low temperature rather than its frequency or time of occurrence is an important factor for fruit formation (Young 1970). In Australia, plant growth is optimum at 30 °C and ceases below 15 °C but flowering occurs at 10 °C. In India, maximum temperature during flowering varies from 21 °C in February to about 38 °C in June (Pandey and Sharma 1989). Wet spring/summer and dry winter conditions are desirable for litchi fruiting in India (Kanwar et al. 1972a). China receives an annual rainfall of 1,500 mm having 69–84% humidity in litchi growing areas that is beneficial for the crop. Sunlight intensity also plays detrimental role in litchi cultivation because intense light in summer causes sun-burning and skin cracking of fruits (Kanwar et al. 1972b).

It is obvious that different soil and/or climatic conditions cover different areas of litchi cultivation. Certain elite cultivars, grown through ages in a specific area, are well adapted to soil and climate of that area. Their propagation in other areas has not been beneficial because the quality of the same cultivar is often affected. For example, ‘Shahi’ litchi of Muzaffarpur in North Bihar, producing the best quality fruits in India, is not able to retain the same quality grown in other areas of Bihar or outside. Besides North Bihar (Ray et al. 1985), the performance of litchi cultivation in other areas varies with agro climatic and soil conditions in Punjab (Jawanda and Singh 1977). Similarly the Chinese recognised the extreme difficulty of maintaining the yield of highly prized cultivars under climatic, soil or cultural conditions other than those of the tree’s original areas of selection (Menzel and Simpson 1987). Thus there is considerable variation in fruit quality that is specific to locality, environmental conditions and cultural operations.

7.1.1 Major Producers

China is by far the largest litchi producing country in the world, with the cultivated area more than 584,000 ha with an annual production of 958,700 metric tons. The most important fruit producing city is GaoZhao (in West Guangdong) near Guaghou (Canton), the very heart of South China on the banks of the Pearl River having boundless expanses of litchi plantations with beautiful dark green dome-shaped canopies (FAO 1990). Other litchi growing areas are Dongguan and Shenzhen, famous for the elite litchi cultivars like the Smile of the Emperor’s Concubine (Fexixiao), Guiwei and Nuomici. Until the year 2001, the growing areas exceeded 584,000 ha. Commercial activity is concentrated in Guangdong, Guangxi, Fijian and

Hainan Provinces with minor share from Sichuan, Yunnan and Guizhou (Huang et al. 2005).

After China, India is the second largest producer of litchi, cultivated area 56,538 ha with an annual production of 474,000 metric tons. According to State Horticulture Department, at least 1,000 ha of cropping areas are added annually in the Bihar State that contributes 77% of total litchi production in India. Tripura, Punjab, West Bengal, Arunachal Pradesh and Karnataka are other litchi producing states of India. The main bulk of the fruit production comes from China, India and Taiwan but it is gaining popularity in several other countries like South Africa, Australia, Malaysia, Burma, Hawaii, USA, Israel, Mauritius and West Indies (Pandey and Sharma 1989). The quality of litchi exported from India is considered to be the best (Table 7.1a and 7.1b).

7.1.2 Consumption

Litchi fruits are largely consumed fresh locally in the areas of production. As their shelf life is very poor, their transport to distant places face an uphill task due to rapid

Table 7.1a Production of litchi in India

Year	Area (ha.)	Production (tons)	Yield (ton/ha.)
1970	1,330	5,320	4
1975	1,876	8,629	4.5
1980	2,522	11,349	4.5
1985	2,702	16,212	6
1990	6,045	36,270	6
1995	7,088	44,643	6.29
1998	7,288	44,653	6.12
1999	7,467	43,804	5.86
2000	7,467	61,000	8.02
2001	7,517	74,000	10
2002	7,667	53,000	6
2003	7,667	75,000	9.7

Table 7.1b Litchi fresh fruits exports

Year	Quantity (tons)	Importing country
1994	10	England
1995	30	France, The Netherlands
1996	Nil	Lack of quality litchi
1997	20	England, France
1998	20	England, France
1999	30	England, France
2000	25	England, France
2001	53	England, France, Dubai(Gulf Country)
2002	42 Litchi Fresh 50 Litchi Juice	Dubai, England Nepal
2003	46 Litchi Fresh 170 Litchi Juice	England, Spain, The Netherlands, France, Nepal

deterioration in bright colouration and flavour within a few days. Several attempts to retain fruit quality even for 7–10 days have not been successful leading to expensive wastage and often resulting in a glut in the market. A good proportion of fruits undergo rotting in India. However, in China dried litchi fruits (as dried nuts) are quite popular and also exported on a large scale to several countries. Canned litchis or flavoured squash or frozen fruits are other modes of preservation and consumption which helps in avoiding a glut in the market and in preventing rooting of the fruits. Frozen litchi fruits retain flavour and quality if the freshly harvested fruits are rapidly cooled and maintained at 25 °C at which they remain in good condition for 12 months (Morevil 1973). If preserved as pulp, they are acceptable for 6 months at 25–35 °C and up to 12 months at 4−5 °C in India (Sethi 1985).

7.1.3 Uses of Litchi

Litchi fruits are among the most delicious having high nutrition and medicinal values. Edible portion of the fruit is a thick, translucent juicy aril with high sugar content that may be as high as 18–20% in elite cultivars (Chadha and Rajpoot 1969), and (Chan et al. 1975). The fruit is an excellent thirst quencher and reported to serve as a tonic for brain, heart and liver (Syamal and Mishra 1989). From the seeds of litchi, ά-(Methylenecyclopropyl) glycine, an analogue of hypoglycine A was isolated that exhibited hypoglycemic activity in animals (Gray and Fowden 1962). In China, litchi leaves are used for making poultices, the seed for skin disease and decoctions of flowers and the bark and the roots for throat gargle (Pandey and Sharma 1989). Besides possessing high sugar content litchi is a good source of ascorbic acid that ranges from 40 to 90 mg/100 g and minerals (0.7%) like calcium, phosphorus and iron (Watt and Merril 1963).

7.2 Origin and Domestication

Litchi, one of the most precious fruit crops, originated in the southern parts of China where it has been cultivated and owes its origin in the Chinese provinces of Kwantung and Fukien (Tao 1955; Ochse et al. 1961). In the words of de Candolle (1909), ‘Chinese knew about litchi only late in the third century of our era’. Though questionable, the first reference to this fruit in literature probably appeared as early as 1766 B.C. However, a clear reference has been mentioned in the literature of the Han dynasty (140–86 B.C.). A monograph by Ts’a’ Hsia ng (A.D. 1059) is possibly the first publication on litchi (Groff 1921), which is a most complete book on litchi in English. Apparently it reached Burma and eastern India by the end of the seventeenth century or shortly thereafter (Hayes 1957). Subsequently it made its way to Bengal by the end of the eighteenth century (Goto 1960; Liang 1981) from where it diversified to other parts of India and was cultivated on a commercial scale.

The introduction of litchi to several other countries has been a later event. In Hawaii, it was probably introduced in 1873 and met with remarkable success. In West Indies its cultivation started by 1775 and in Natal (South Africa) in 1869 (Marloth 1947). Although introduced in Queensland (Australia) as early as 1854, its cultivation on commercial scale was of late occurrence (Batten and Lahav 1994). In the USA it reached from Saharanpur (India) to Florida in 1883, in California in 1897 and subsequently again to Florida from Fukien province of China in 1906. The variety was named as 'Brewster' litchi. Early in the nineteenth century it also reached England and France but failed to establish (Pandey and Sharma 1989).

7.3 Botanical Aspects and Systematics

L. chinensis (Goertn.) Sonn. belongs to the family Sapindaceae, under sub-family Napheleae. Several synonyms are known namely, *Euphoria litchi* Desf., *E. sinensis* Gmel., *E. punicea* Lamk., *E litchi* Juss., *Dimorcarpus lychi* Lour., *D. litchi* Will., *Nephelium dimocarpus* Hf. and T., *N. duriocarpus* T., *N. litchi* Camb., *Sapindus edulis* Ait., *Scytalia chinensis* Goertn, *S.litchi* Roxb, *S. locacon* Roxb. and Litchi *litchi* Britton. Although belonging to the soapberry family, Sapindaceae contains 1,250 genera and about 1,000 species, mostly consisting of tropical and subtropical fruits; only a few plants can truly be termed as horticultural crops. Among them, litchi is the most important while other members are longan (*Euphoria* longana Lamj.), rambutan (*Nephelium* lappaceum L.) and pulsan (*N. mutabile* Blume). Some other species of less horticultural interest but related to litchi growing outside India are *Blighia sapida* Koen, *Melicocca bijuga* L., *L. phiippinensis* Radlk., *Pseudonephelium fumatum* Radk. and various species of Euphoria (Groff 1921). *L. chinensis* Sonn. has various common names, namely, litchi, lychee, lici, li-ci, leechee etc. (Hayes 1957). In Thailand it is called lin-chi while Malayan names are kalenkeng, lingking, laichi etc. (Allen 1967). In China it is termed as lizhi (Paull and Chen 1987). Litchi tree is a mycorrhizal round topped, 10–15 m high, evergreen tree with spreading branches. Leaves are petiolate, exstipulate and paripinnately compounds having 2–4 pairs of 8–15 cm long leaflets that are coriaceous, elliptic and oblong to lanceolate, shortly acute, glabrous and shining above. Flowers grow in terminal panicles and are polygamous, regular, small and inconspicuous having greenish white or yellowish colour. Sepals are small and valvate. Petals are often wanting. Stamens are usually eight with hairy filaments that are incurved in bud but straight, erect and far exerted later. Ovary is two-lobed and compressed silky, only one lobe usually develops into fruit. Stigma is bilobed. Fruits grow in loose bunches of 2–20. Each fruit is oval in shape, about 3.8 cm in diameter. Ripe fruits possess dry, brittle, tubercled pericarp. The colour of the ripe fruit, which is likened to a large strawberry in appearance, is rose – red to deep red and the colour changes to dull brown as the fruit dries. Botanically, the fruit is a nut but in possession of a white, translucent, fleshy and juicy aril (the edible part) developing from the funicle

and surrounding the seed at maturity makes it fleshy. The aril is of firm texture with sub-acidic flavour. On drying, the aril shrinks away from the thin outer shell (the pericarp) remaining as a rather tough structure around the seed and possess a flavour of raisin, bearing no resemblance to that of a fresh delicious ripe fruit (Pandey and Sharma 1989).

7.4 Cytogenetics

Reports on the cytogenetics of litchi have been scanty (Liu 1954; Chapman 1984b). This aspect has not received the proper attention it deserves with an aim to evolve new cultivars. The species probably originated through hybridisations of more than one wild progenitor. Haploid chromosome numbers 14, 15, 16 and rarely 17 were reported and variable chromosome number pointed to multiple progenitor origin. Liu (1954) considered the so-called 'mountain litchi' with inferior fruit quality clearly distinct and more resistant to frost than the elite cultivars existing today.

7.5 Reproductive Biology

In litchi floral axis is of compound racemose type but the flowers occur in cymes. On the basis of sex, groups of flowers in a cyme have been different and six types of cymes are noted. They are: (i) staminate flowers (ii) pistillate flowers (iii) terminal staminate and lateral pistillate flowers (iv) terminal pistillate and lateral staminate flower (v) terminal staminate with lateral flowers of different sexes and (vi) terminal pistillate with lateral flowers of different sexes. Three types of flowers are present in different branches of panicles on the same tree. They are: (a) hermaphrodite with abortive ovary, that is, functionally male; (b) hermaphrodite with non-dehiscent anthers, that is, functionally female and (c) male (Singh and Dhillon 1983). The duration of anthesis is usually between 20 and 45 days (Chadha and Rajpoot 1969; Pivovaro 1974). However, in Punjab (India) anthesis has been reported to continue for 11–17 days with optimum anthesis in the forenoon (Singh and Dhillon 1983). Anther dehiscence has been reported to begin the day after anthesis and continue up to 3 days but all the anthers do not dehisce simultaneously (Chaturvedi and Saxena 1965). In dry condition, pollen grains are barrel-shaped and tend to be triangular when mounted on a slide in water or lactic acid (Banerjee and Chaudhuri 1944). They are bi- nucleate at the time of shedding. Pollen grains of male flowers are less viable than from anthers of hermaphrodite flowers (Mustard et al. 1953) and in their germination on artificial media supplemented with sucrose, auxin and boron (Shukla et al. 1978).

The flowers are bicarpellary syncarpus with a bilobed superior ovary. The fertile lobe rapidly increases in size and turns erect and the other lobe is usually abortive. Style remains erect between ovary lobes having terminal bifid stigma with revolute branches. There is one anatropous ovule in each locule of the ovary. Functionally, pistillate flowers bloom. At the time of its lobe initiation stigma becomes receptive

exhibiting 75% (maximum) receptivity usually a day after anthesis that continues up to two additional days (Chaturvedi and Saxena 1965). Litchi flowers are self-sterile. Nectary glands are present and pollination is entomophilius. Butcher (1957) recorded 27 species from Florida, USA. *Callitroga macellaria* (a screw worm of the dipteran order) was observed to be the most effective pollinator whereas *Aphis dorsata*, the honey bee and Coleoptera, Hemiptera, Homoptera and Lepidoptera are other effective insects. In India, *Apis* spp. and *Melipona* spp constitute 98–99% of total pollinators (Pandey and Yadava 1970).

Several reports on fruit development of various litchi cultivars are available (Gaur and Bajpai 1978). One of the two locules of the ovary develops into a fruit, the other locule being shriveled and persistent as an appendage at the base of the fruit. Occasionally both locules develop into fruits. Prasad (1977) observed both locules developing into fruits in small proportions in litchi cv. 'Deshi', 'Kasba' and many other cultivars growing at Sabour, Agricultural College garden in Bihar, India. Small, immature fruits have a green velvety appearance that later turn into tubercles (epicarp tissue) at maturity (Banerjee and Chaudhuri 1944; Pandey and Sharma 1989). Within the fruit a single seed develops from an anatropous ovule after fertilisation. At the beginning, the seed is small and light green in colour that develops into a large chocolate colour at maturity. During the course of maturation, an aril emerges from the base of the seed (Huang and Xu 1983) and gradually surrounds the entire seed in fully mature fruit. The fleshy aril is translucent, white and becomes juicy with the advancement of fruit ripening (Prasad 2000).

Litchi seed is recalcitrant in nature and sensitive to moisture stress (Chin et al. 1984, Ray and Sharma 1989, Fu et al. 1990; Kumari-Singh and Prasad 1991). Within the fruit, seed viability is maintained but is rapidly lost within a day or two after separation from the whole fruit (Xia et al. 1992a and b; Prasad et al. 1996). Seeds sown soon after separation from the whole fruit, germinate readily and the optimum conditions required for germination are sand bed under shade with regular irrigation. In a litchi cultivar, 'Early Bedana' with a chicken tongued seed, traditionally considered non-viable (Pandey and Sharma 1989), germination rate was as high as 60% when sown in the sand bed under the shade (Prasad et al. 1996).

7.6 Fruit Growth Characteristics

Several reports have appeared on litchi fruit growth. Kanwar et al. (1972a) studied fruit growth at Guardaspur (Punjab, India). Singh (1977) as well as Gaur and Bajpai (1978) reported some aspects of development in a few litchi cultivars. Jaiswal et al. (1982) observed fruit growth pattern of five litchi cultivars, namely, Purbi, Deshi, Green, Ajhauli and Kasba from the orchard of Sabour Agricultural College in Bihar. They examined the whole fruit and fruit parts including the rind (pericarp), aril and the seed of various cultivars. Works of Huang and Xu (1983) as well as Huang and Qiu (1987) in Chinese cultivars 'Nuo Mi Ci', 'Huai Zhi Wei' and 'Xiang Li' recorded growth curves of whole fruit and fruit parts to be typically sigmoid,

which is consistent with earlier reports (Kanwar et al. 1972b, Gaur and Bajpai 1978; Jaiswal et al. 1982). According to these authors, the aril development depends more upon the rind than the seed coat and is repressed by a rapidly growing embryo within the seed. This explains why large seeded fruits possess a small proportion of the aril.

7.7 Conservation of Germplasm

Litchi has been under cultivation since long, but most of the commercial cultivars have been selected either in Chinese or Indian conditions resulting in their adaptation to limited climatic conditions, outside which cultivation is not as successful. Groff (1943) considered litchi to be an ecotype on account of this fact. Excessive domestication through ages poses a threat to their survival under continuously changing climate of the world. However, rapid advancement in the field of seed technology and biotechnology appears to have revolutionised the germplasm conservation in such a way that seeds or various plant parts are stored indefinitely. Cryopreservation, the technique of storage of biological material at −196 °C in liquid nitrogen is being used for long-term storage of plant germplasm that can be utilised in future breeding programmes undertaken at the National Plant Tissue Culture Repository (NFPTCR) of the National Bureau of Plant Genetic Research (NBPGR). Several laboratories in the world have standardised protocols for seed storage at −196 °C. Besides seed banks, *in vitro* gene banks have also been established in an effort towards germplasm conservation. The *in vitro* technology offers an efficient means of storing vegetative propagated recalcitrant seeds, excised embryos of which are much more tolerant to desiccation as in cases of rambutan, *Nephelium lappaceum*, closely related to *L. chinensis*, having better prospects of their storage at low temperature even cryogenically with the help of cryoprotectants (Chin and Hor 1989). Cryopreservation thus offers an alternative of seed storage to storage of tissues and excised embryos, more so in cases of recalcitrant seeds and vegetative propagated plants. In order to identify genetic materials containing useful traits for breeding and germplasm enhancement, a systematic evaluation of genetic diversity is needed for understanding relationship among accessions and their collecting site environment (Steiner and Greene 1996). Understanding the genetic diversity within a germplasm collection facilitates its use (Strauss et al. 1998). Germplasm enhancement and utilisation are important parameters for genetic improvement by breeding.

7.8 Nomenclature of Cultivars

Numerous cultivars are known throughout the world on the basis of morphological traits which have limited utility in their identification due to environmental interactions (Nielson 1985). It is but natural to have genetic markers for nomenclature of litchi cultivars that in its present state suffers from many inconsistencies, namely,

the same cultivar may be known under several names and different cultivars may appear under the same name (Aradhya et al. 1995). For instance, the cultivar 'Fay Zee Siu' of China is named 'Yu Her Pau' in Taiwan (Menzel and Simpson 1997) whereas 'Emperor', of Florida is called 'Chakrapad' in Thailand (Subhadrabandhu 1990). Isozyme analysis revealed the first two cultivars to be the same and the last two to be identical in all the enzyme systems examined (Degani et al. 1995). In fact they, for the first time, demonstrated isozyme polymorphism to be useful in proper characterisation of 30 litchi cultivars. As cultivars are ill defined, they need proper investigation.

The chief commercial cultivars are Hei Ye, Nuo Ml Ci or No Mai chee (called Groff and Hak Yip elsewhere), Huai zhi, Gui wei, Chen zi (called Brewster elsewhere), Xiang Li and Fay Zee, Siu (all Chinese cultivars); Mauritius from Israel, Chakrapad and Hang Huang from Thailand; Yu Her pau from Taiwan, HLH Mauritius from South Africa; Emperor Black Leaf Haak Yip, Bengal and Brewster from USA; and Kaimana, Sah Kang and Haak Yip from Australia. Elite cultivars of India are Shahi, Early Bedana, Late Bedana Deshi, Early Large Red Purbi Ajhauli, China, Green, Rose Scented, Dehra Rose Madrazi, Kasaili, Calcuttia, Kasba and many others (Pandey and Sharma 1989). From the overview of the traditional cultivars it is clear that their nomenclature is ill defined and without any scientific validity. For example, Aradhya et al. (1995), while investigating genetic diversity of 49 litchi accessions on the basis of comparison of isozyme finger prints, reported that some accessions identically named such as No mai tsz, Kwai mi and Hak Ip possessed different isozyme genotypes while some others with different names displayed identical isozyme genotypes. Comparison of parents using difference in DNA markers may be one of the methods with which breeders can enhance the probability of selecting those parents with different gene set. Such a method has the potential of producing progeny with new and more favourable combinations of genes for quality and yield (Kumar et al. 2006). DNA based markers like random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) therefore have come under much emphasis in order to evaluate genetic diversity of exotic litchi cultivars in several countries like China (Ding et al. 2000), Thailand (Tongpamnak et al. 2002) and India (Kumar et al. 2006). The latter found 2 out of 27 accessions, Chinarose and Late Bedana, to be genetically distant from other Indian accessions. This would be useful in mapping litchi genome and also in classical breeding.

7.9 RAPD Markers and Genetic Relationships

Compared with most tropical fruit species, genetic diversity of litchi is limited. Most varieties originated and were selected in China and are vegetative propagated. Genetic diversity in litchi is indicated in occurrence of a large number of cultivars in India and China that provide the bases for development of new

cultivars. The environment profoundly influences cultivar characteristics and this may explain why a large number of cultivars are available (Groff 1921). Litchi grown in northern parts of India, namely, states of Punjab, U.P. and Uttaranchal, where climatic conditions (low humidity, low temperature and basic soil) are different from the main litchi growing region of Bihar (India). In spite of different environmental factors litchi cultivars do not exhibit differences in flowering and harvesting time. There are different characteristics, which are used to identify the cultivars. The size and shape of litchi fruit are characteristic for different cultivars (Galan 1989). A study of 11 litchi cultivars for 8 fruit quality traits at Kalyani in the State of West Bengal (India) revealed genetic diversity and the cultivars fell into two clusters. Inter-cluster cultivar crossing might lead to heterosis for fruit trials (Dwivedi and Mitra 1995). Recently, Lin and Mei (2005) used 60 litchi cultivars, one longan cultivar and one tentative inter-generic hybrid of litchi and longan in a study of their genetic relationships and succeeded in constructing a phylogenic tree based on 470 RAPD loci amplified from 30 random primers. Their results revealed that four pairs of different cultivars, prevalent in China since long, were synonymous.

Kumar et al. (2006) reported first the genetic relatedness among Indian litchi (*L. chinensis* Sonn.) cultivars using RAPD markers. Fourteen RAPD primers that produced consistent profiles were chosen, resulting in amplification of 77 reproducible polymorphic bands. The RAPD analysis produced an average of 15.8% polymorphic and 0.10% monomorphic markers. Using the RAPD markers, all the accessions were classified into different groups despite their same or different geographical origins and climatic adaptations (Fig. 7.1). The polymorphism information content scores were calculated for each of the 77 RAPD polymorphic fragments using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Dendrograms using Jaccard's coefficients reflected no clear-cut variation or grouping based on either morphology or climatic adaptation. However, dendrogram showed that 27 accessions of Indian litchi could be classified into groups when the similarity coefficients were shown in a range of 0.11–0.47.

Two accessions (LH80 and LH109) were genetically very far distant from the other accessions using both types of markers. RAPD and AFLP marker analyses provided a quick and reliable alternative for identification of litchi accessions and determination of genetic diversity among them. Khurshid et al. (2004) reported the genetic diversity in different morphological characteristics of litchi. Genetic diversity in morphological characteristics of four litchi cultivars growing under the agroclimatic conditions of Multan was studied. Various characteristics like tree height, canopy spread, tree shape, foliage texture and colour, leaf length, width, shape and orientation, inter-nodal distance, number of leaflets per leaf, number of leaves per flush, flush colour, panicle length, number of anthers and carpels per flower, filament and style size, fruit colour and size were taken into count and variation in the characteristics was discussed. The cultivars differed in some of the morphological characteristics. The differences were probably due to their genetic make up as well as due to the influence of climatic factors. According to Tongpamnak et al. (2002) the genetic diversity and relationships within Thai litchi cultivars was

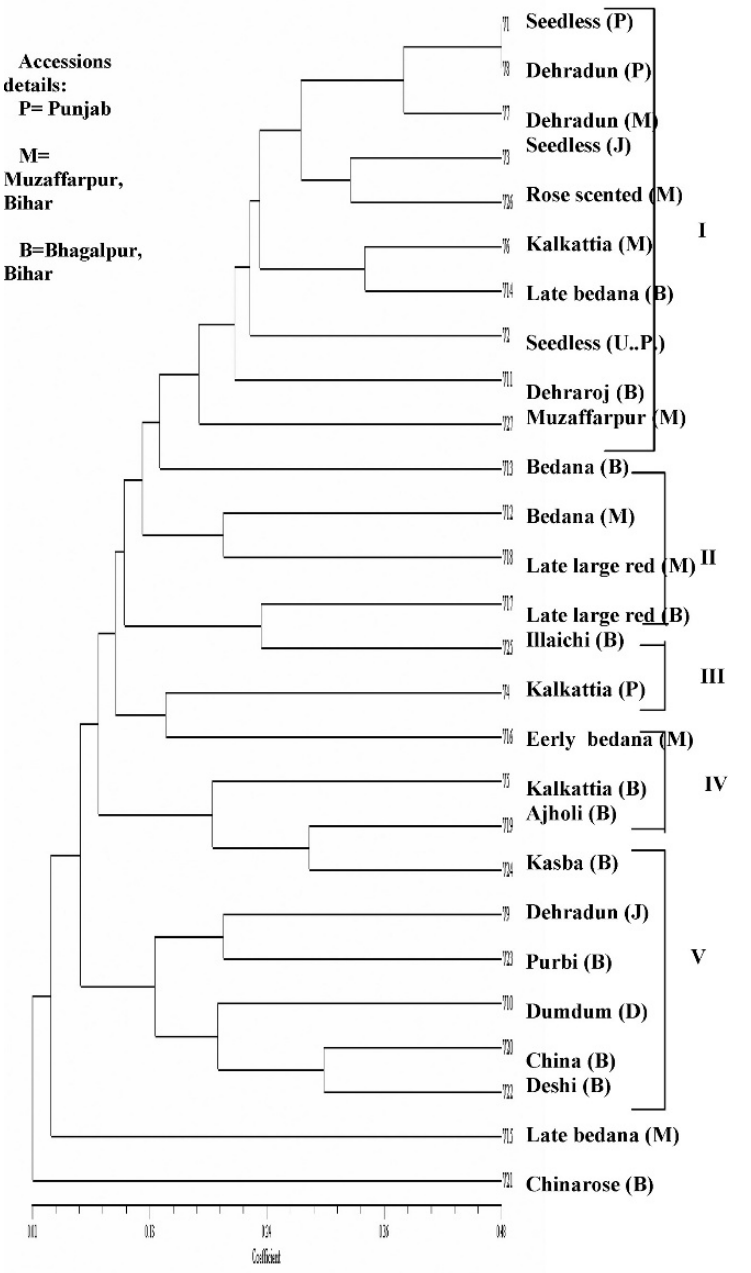

Fig. 7.1 Dendrogram derived from UPGMA cluster analysis using disc coefficient of RAPD markers

investigated using RAPD and AFLP markers. Fourteen RAPD primers and seven AFLP primers were chosen, resulting in amplification of 52 and 101 reproducible polymorphic fragment products, respectively. The percentages of polymorphic markers for RAPD and AFLP were 34.6% and 36.3% respectively. Each marker system was able to differentiate all accessions. Each of the AFLP primers could also identify all accessions, while the RAPD markers did not show such efficiency. The polymorphism information content (PIC) scores were calculated for each of the 52 RAPD and the 101 AFLP polymorphic fragments. It ranged between 0.16 and 0.50 for RAPD markers and 0.22–0.50 for AFLP markers. Anuntalabhochai et al. (1999) reported an analysis of genetic variation within 20 litchi cultivars using random amplified polymorphic DNA (RAPD) technique. Out of the 69 arbitrary primers, 5 primers named OPB18, OPC09, OPAK10, OPAQ12 and OPAS10 produced reliable DNA polymorphism ranging in molecular weight from 200 to 2000 bp. DNA patterns from RAPD data were analysed by cluster analysis and UPGMA to present a dendrogram depicting the degree of genetic relationship among the 20 cultivars. These cultivars were classified into two major groups with each containing three sub-groups. Analysis revealed that some cultivars known as O-Hia and Haak Yip displayed identical fingerprint patterns indicating that it is the same cultivar known under two different names. Others such as Kwang Jao and Jean Hom, and Haew and Luk Lai exhibited high similarity in their patterns indicating a close genetic relationship.

Ding et al. (2000) studied the segregation patterns of RAPD markers in an F_1 population of *L. chinensis* Sonn. F_1 population was established from a cross between cultivars Wuye and Luhebao of *L. chinensis* Sonn. using the pseudo testcross format. Twenty nine, 10-mer primers were screened out of 75 primers and 294 RAPD markers were amplified from 68 F_1 seedlings. Of these 53 polymorphic markers, 36 segregated in a ratio of 1:1 (band present; band absent). Among these 36 markers, 21 were present only in 'Wuye' and 15 only in 'Luhebao'; 17 markers segregated at a ratio of 3:1 (band present: band absent) and 232 markers did not segregate in F_1 (the parent genotypes may be AA × AA, AA × Aa, Aa × AA, aa × AA or AA × aa). Additionally, 7 markers deviated from Mendelian Law and two showed abnormal segregation patterns. Of the 146 markers present in 2 parents, 123 bands (84.2%) did not segregate and 17 bands (11.6%) segregated normally (3:1). Of the 148 markers present in only a single parent, 14.2% were in 'Wuye' and 10.2% in 'Luhebao'. The information obtained from the molecular marker analysis could be of practical use in mapping the litchi genome as well as for classical breeding.

7.10 Problems Facing Litchi Production

Major bottlenecks that limit fruit production are listed below and they need careful attention:

7.10.1 Lack of Quality Seeds and Genuine Plant Material

Because of long gestation period, high heterozygosity, lack of information on inheritance pattern, infection of seeds and inadequate supply of genuine and certified plant material to the growers are some of the reasons for the low productivity of fruit crops. In other perennial fruit crop like mango, plant multiplication is being done by grafting techniques on nondescript root stock resulting in inferior plants. Similarly, litchi crop is being multiplied through stooling, air layering and budding that are sluggish and cumbersome and the plants are not multiplied through elite 'mother of superior' quality. Therefore, multiplication should be done only from the mother plants of established superiority. It would be desirable to establish elite orchids of important fruit crops in the fruit growing state for the supply of authentic plant materials.

7.10.2 Constraints in Micro-Propagation

In vitro oxidative browning of cultures, contamination, vitrification and high mortality during acclimatisation are some of the problems associated with micropropagation of woody fruit trees. *In vitro* oxidative browning can be controlled using different phenol binding agents, modifying redox potential of media, quick sub-culturing, keeping cultures in the dark and through explant waxing (George and Sherrington 1984, Mishra and Mishra 1999). The conditioning of stock plant has been very important for the establishment of shoot tips and nodal explants. New vegetative growth was found to be used as an explant in jackfruit, guava (Amin and Jaiswal 1988) persimmon (Mishra 1982) and a number of other fruit crops (Amin 1992). Substantial numbers of micro-propagated plants do not survive from in vitro condition to green house or field environment. The plantlets develop within the culture vessels under low levels of light, aseptic conditions on medium containing ample sugar and nutrient to allow for heterotrophic growth and in an atmosphere with high relative humidity.

7.11 Litchi Agronomic Practices

7.11.1 Cropping Systems

A common practice in litchi orchard is pruning of young trees in order to establish a strong framework that will facilitate harvesting later. Young trees are sensitive to intense heat, frost and high winds. They require moist soil with regular irrigation for their active growth. Mature trees require regular use of fertilisers (Pandey and Sharma 1989). Trees are tinctured after completion of post-harvest flush. Maintenance of good sanitation and weed control are essential factors for keeping a litchi orchard in healthy and disease free atmosphere. Since litchi is a slow growing

tree and takes about six years to come to the bearing stage, inter-cropping of a young orchard during the vegetative period is a widespread practice in all litchi growing areas. Chaturvedi and Jha (1998) studied in detail the crop production and economics under litchi plantation across 1–9 year age series in North Bihar, India. They used inter-crops under two rotations, namely, paddy-wheat-green gram, ginger-maize, maize-mustard etc. under 1–9 years of litchi plantations.

7.11.2 Watering

Young orchards require regular irrigation otherwise plants will not be established properly and the growth is affected. Adequate watering is required at the fruit bearing stage. It is essential to maintain optimum soil moisture content (Cull and Hams 1974). However, if annual rainfall is more than 125 cm and well-distributed, irrigation may not be needed (Pandey and Sharma 1989). In India, critical period for irrigation is from January end to monsoon break (June end/July beginning). Chapman (1983) suggested that irrigation in Queensland (Australia) needs to be increased from the time of panicle emergence to the fruit harvest in order to promote floral and fruit development as well as initiation of post-harvest vegetative flush. Chandel et al. (1995) investigated the effect of irrigation frequencies on yield and quality of 26-year-old litchi cv. Rose Scented growing in Nainital (India) and reported highest yield of 78.5 kg/tree following 7 irrigations at 15 day intervals with low incidence of fruit cracking (6.69%) . In another cultivar Dehradun highest fruit set (14.36%), fruit retention (9.98%), fruit size, weight and yield (64 kg/tree) was recorded from trees sprayed with NAA (1-naphthalenacetic acid) and irrigated at 20% depletion of available soil moisture .Though litchi is a deep rooted fruit tree, absorbing roots mostly exist in the topmost layer of the soil at 20–30 cm depth that must be maintained at 50% soil moisture or above (Cull 1977). A calendar of operations all year round for irrigation and use of fertilisers besides growth regulators has been prescribed by Pandey and Sharma (1989).

7.11.3 Fertilisers

Nutritional requirements and use of fertilisers formed the basis of major thrust of an earlier research on litchi. It was established that NPK application increased fruit yield (Koen et al. 1981a and b) and their deficiency resulted in stunted growth and floral initiation. Micro-nutrients like zinc, boron and copper are very important for litchi nutrition. For plantation in the orchards, digging open pits of about 2–3 weeks beforehand and filling with a mixture of rotten farmyard manure silt is followed by filling with a mixture of farmyard manure (20–25 kg), bone meal (2 kg) and potassium sulphate (400 g) to be mixed with top soil in the pit. It is thus clear that the nutritional requirements for litchi plantation are high. Koen et al. (1981b) recommended the highest yield with 1,200 g nitrogen per tree initially to be increased

to 3,600 g nitrogen later. For mature trees a mixture of calcium ammonium nitrate (4 kg), super phosphate (3 kg) and potassium chloride (1.5 kg) per tree per year was also recommended (Koen and Smart 1982). However, in India little or no manure was applied earlier (Hayes 1957) but Yamdagani et al. (1980) recommended 1.0 kg nitrogen, 300 g phosphorus, 300 g potassium and 40 kg FYM per mature tree per year. Thus practice of fertiliser application has been different at different places. In recent years various trials of fertilisers have been undertaken in order to maximise fruit yield and rapid healthy growth of litchi trees. Field experiments on 21-year-old litchi trees were conducted and application of N, P and K at 0.84, 0.5 and 1.2 kg per year respectively increased the yield (Chen et al., 1998). At Pantnagar in India, application of 1,200 g nitrogen per tree with 300 g per year and potassium resulted in the highest yield and improvement of fruit quality (Lal and Tiwari 1996). Likewise, Sharma and Mahajan (1997) made a critical appraisal of fertiliser requirements during the years 1975–1995 at Gurdaspur (India). Hasan and Chattopadhyay (1993) observed change in growth and fruit quality of litchi cv. Bombai when put on various trials of NPK nutrition.

7.11.4 Pest Control

A considerable fruit loss (3.6%) is caused by insects and birds. Out of the 40 insect species reported (Vevai 1971), two insects, namely, eriophyid mites (*Aceria litchi*, syn. *Eriophyes litchi*) and bark eating caterpillars bring about serious damage (Butani 1977). Other important insects are scale insects, leaf miners, bugs, weevils, fruit and seed borers, etc., that occasionally infest. Eriophyid mite is widespread in litchi growing countries but its incidence is maximum in North Bihar and Mysore (India). Besides infestation of leaves, it causes inflorescence malformation (Das and Chowdhary 1958). Several control measures and insecticide sprays are in practice that has been dealt in detail by Pandey and Sharma (1989). Important bark eating caterpillars like *Indarbela quadrinotata* Walker and *I. tetraonis* More and some other bark borers are known to cause damage (Chang 1970; Villiers and Mathee 1973; Rai and Bhandary 1973). Cleaning the affected portion after removal of webs formed and plugging the holes with cotton wool soaked in carbon bisulphide, chloroform, formalin or petrol and finally with mud has been an ineffective control measure against the insect pests. Low concentrations (0.05%) of dichlorofos and p-sulfan or parathion (Villiers and Mathee 1973) are also effective. In addition to these control measures, exploitation of proteinanceous enzyme inhibitors in integrated pest management, as in other crops, appears a sound future prospect that emphasises the need for development of transgenic litchi trees resistant to insect pests. Not only genes encoding proteins and amylase inhibitor have been isolated and cloned, transgenic crops using these genes have been developed (Chrispeels et al. 1998; Ussuf et al. 2001). Hopefully this strategy can be exploited in litchi in the near future.

7.11.5 Diseases and Control

Numerous reports have appeared on incidence of diseases caused by fungal pathogens after fruit harvest in several countries (Pandey and Sharma 1989) and Underhill et al. (1997). *Collectotrichum* and *Phomopsis* sp. are reported to infect fruits in the field before harvest (Johnson and Sangchote 1994). Some of the post-harvest pathogens include several species of *Aspergillus*, *Pencillium, Botryodiplodia*, *Pestalotiopsis, Fusarium, Trichoderma* etc. Yeasts and bacteria are also reported (Roth 1963) to attack litchi. Duvenhage (1993) reported control of post-harvest decay and browning of litchi fruits by sodium metabisulphite dip followed by hydrochloric acid dip or vitafilm. Several fungicides like benomyl, prochloraz and imazalil are effective in disease control (Pandey and Sharma 1989; Underhill et al. 1997). In addition to the use of fungicides, refrigeration, heat treatment and orchard hygiene are other control measures. Post-harvest handling at low temperature minimises disease occurrence. Incidence of red rust is caused by parasitic alga *Cephaleuros virescens* on stem and leaf of litchi, causing loss of vigour due to bank canker and brown leaf felting. Gupta et al. (1997) recorded it in litchi orchards in the Kangra Valley of Himachal Pradesh (India) at an altitude of 750–950 m and infestation ranged from 50 to 90%. Four sprays of dimethioate (0.03%) or dicofol (0.05%) at monthly intervals followed by pruning and burning of affected parts is an effective control measure.

An entophytic fungus *Phomopsis litchii* has been reported and its occurrence poses problem for *in vitro* culture. This could be controlled by including bavistin, a broad range fungicide in medium bavastin (Kumar 2006). Development of disease resistance through biotechnology is a major potential area that needs to be exploitated in future for preventing expensive wastage of the plants as well as the fruit.

7.12 Breeding Objectives and Strategies

7.12.1 Breeding Objectives

Litchi is a cross pollinated crop with a high degree of heterozygosity that puts constraints on developing plants through sexual means. Most of the commercial cultivars selected under Chinese or Indian conditions have been adapted to limited climatic conditions (Pandey and Sharma 1989). Characters like fruit size, quality and period of maturity formed the basis of cultivar selection, raised through asexual means. However, in order to diversify litchi cultivation, in addition to these, other characters like precocity, dwarfness, regularity of bearing, wider adaptability tree characters and resistance to physiological disorders are of paramount importance. Raising plants through asexual means generates plants that are true to the parental types but variations are not obtained. Variations would arise only through sexual

reproduction and new variants would provide the basis for new selections through genetic manipulation. Thus there is urgent need for breeding work and raising plants through seeds. Survey of literature points to the fact that no attention has been paid to the breeding work for raising new varieties except for small selections for which programmes were initiated sporadically in Hawaii (Storey et al. 1953), Queensland, Australia (Cull 1977) and Saharanpur (India) (Lal and Nirwan 1980). As a result, important cultivars like 'Groff', and 'Brewster' were developed. Undertaking a breeding programme on a large scale needs a comprehensive survey of various genotypes and their inheritance pattern because of obvious difficulties in litchi breeding (Hamilton and Yee 1970; Menzel 1985).

7.12.2 Breeding Strategies

Different breeding strategies are applied for developing new litchi cultivars with improved traits. It takes a long time to develop a new litchi cultivar by conventional breeding. Therefore, it is desirable to reduce the breeding time in producing new improved cultivars. Breeding methods combined with new technologies including genetic engineering, *in vitro* mutagenesis and molecular assisted breeding would assist litchi breeders to develop new cultivars in a cost effective manner.

7.12.2.1 Conventional Breeding

There are many serious problems associated with raising plants through seeds, such as loss of seed viability and its short life span, slow seedling growth and long juvenile period of vegetative growth. In the last 20 years breeding programmes have been undertaken on a large scale in several countries. At the Institute for Tropical and Subtropical Plants (ITSP), South Africa, a breeding programme was initiated during 1992–1993 for the purpose of cultivar selection for South African conditions, aiming at an annual establishment of at least 1,000 seedlings for evaluation and selection (Froneman and Oosthuizen 1995). In China, breeding and selection for extension of the bearing period and better quality cultivars has been initiated (Huang et al. 2005). A seedless litchi variety produced by conventional breeding in Hainan (China) (Fig. 7.2).

In Australia, genetic improvement of litchi has been reported through reciprocal controlled crosses among several existing cultivars (Dixon et al. 2005). A majority of the seeding trees came into production within three years when planted in subtropical and tropical conditions and one notable performer produced 32 kg of fruits with individual fruit weight being 35–45 g. Four new litchi selections were recommended for cultivation in Hainan province of China. Out of these, Aili is a dwarf selection producing fruits of 24.8 g (average weight) whereas another selection Ziangxi has large fruits (39.1–595 g) of very good eating quality. Thus breeding for selection of new litchi cultivars has been receiving the attention of researchers and it is possible that breeding strategies may succeed in control of browning so that the bright red colour of the litchi fruit can be retained (Underhill et al. 1997).

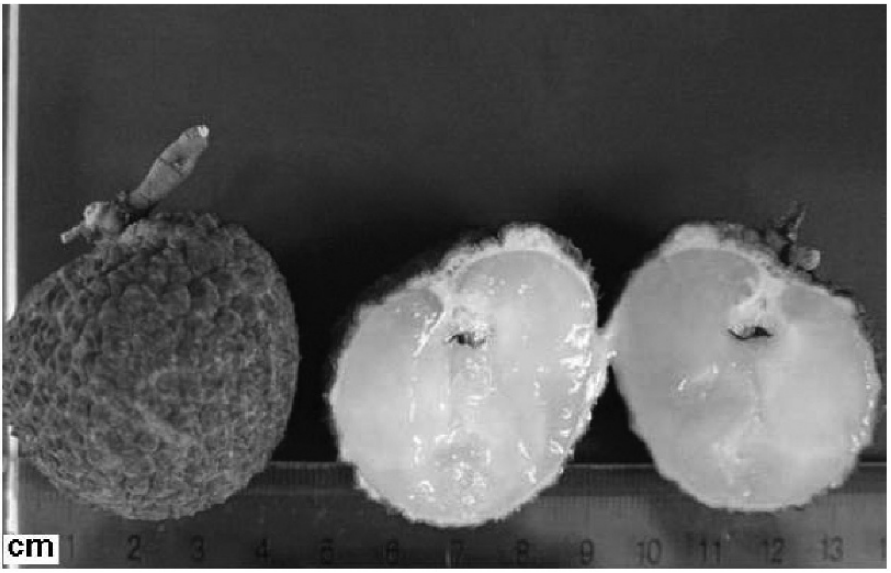

Fig. 7.2 A seedless litchi cultivar in Hainan, China (Photo provided by Prof. Wang, China) (*See* Color Insert)

Other important areas of interest are extension of the bearing period, irregular bearing, poor shelf life and disease resistance. These need to be addressed in future programmes on litchi breeding.

7.12.2.2 Transgenic Approach

There is one recent report on transformation of litchi. Puchooa (2004) reported Green-fluorescent protein (GFP) gene expression in leaf tissues of litchi after transformation using *Agrobacterium*. *In vitro* grown leaf tissues were used for transformation. After four weeks in culture, expression of GFP was apparent when the regenerated callus and the leaves were observed under fluorescence microscope fitted with a blue exciter filter, a blue dichroic mirror and a barrier filter. Although no transformed litchi plantlets were regenerated, screening for GFP gene expression may prove useful to improve transformation efficiency and to facilitate detection of transformed litchi plants. Although Ouyang and Zheng (1985) reported T-DNA transfer and tumor formation induced by *Agrobacterium tumefaciens* on litchi, genetic transformation of litchi using *Agrobacterium* could be a reality in the future.

7.12.2.3 *In Vitro* Mutagenesis

Nuclear applications in food and agriculture have contributed greatly in enhancing agriculture production of seed and vegetatively propagated crops (Jain 2005).

Even though nuclear technology has benefited agriculture greatly, it still has a great potential in genetic improvement of litchi and other crops. More than 2,600 mutant varieties have officially been released in many countries (http://www-mvd.iaea.org). Both chemical and physical mutagens are used to induce mutations. Among them, gamma rays and ethyl-methane sulphonate (EMS) are widely used for mutation induction. Fine embryogenic cell suspension cultures are most suitable for inducing mutations by transferring to the filter paper and plated on the agar-solidified culture medium for gamma irradiation. Initially LD_{50} dose is determined, which is used as an optimal dose for mutation induction. Irradiated cells are further cultured to the fresh medium for the development, maturation and germination of mutated somatic embryos. This approach provides mutated somatic seedlings in a short period and also prevents chimeras problem that otherwise requires to multiply plants up to $M1V_4$ generation for chimera dissociation. Alternatively, shoot tip or bud wood can be irradiated and multiply plants up to $M1V_4$ generation for producing pure mutants by dissociation of chimeras.

The genetic improvement of litchi for improving or developing new varieties requires genetic variation. However, the desirable genetic variation is most often lacking and that hampers the breeding of litchi. This is because the existing germplasm fails to provide the desired recombinants and it is necessary to resort to other resources of variation. Since spontaneous mutations occur with extremely low frequency, mutation induction techniques provide tools for the rapid creation and increase in variability in crop species. The genetic variability can be induced by mutagenic agents, such as radiation and chemicals, and from which desired mutants could be selected (Jain 2006). The mutagen treatment breaks the nuclear DNA and during the process of DNA repair mechanism new mutations are induced randomly and heritable. The changes can occur also in cytoplasmic organelles and also result in chromosomal or genomic mutations and that enable plant breeders to select useful mutants such as flower colour, flower shape, disease resistance and early flowering types (Jain and Maluszynski 2004). A specific advantage of mutation induction is the possibility of obtaining unselected genetic variation, improvement of vegetatively propagated plants when one or few characters of an outstanding cultivar are to be modified.

7.12.2.4 Molecular Marker Assisted Selection and Breeding

As mentioned earlier, nomenclature of litchi cultivation suffers from many inconsistencies that need scientific attention (Aradhya et al. 1995). While the isozyme pattern was used as a reliable marker, rapid developments in technology led to the introduction of DNA based molecular markers that have proved to be very efficient. Among these, RAPD and AFLP, markers are quite beneficial for assessing genetic diversity. In recent years, RAPD markers have been successfully exploited in litchi (Ding et al., 2000; Tongpamnak et al. 2002; Kumar et al. 2006). Comparison of parents displaying differences in DNA markers may be useful for breeders in selection of parents with different genomes resulting in new combinations of characters in their progeny.

7.13 Propagation

Excellent reviews are available on various aspects of propagation (Menzel 1985; Pandey and Sharma 1989) including micro-propagation (Sarin and Prasad 2003; Sarin et al. 2003) in litchi. High heterozygosity and genetic diversity as a result of cross pollination put constraints in practicing propagation of elite cultivars of litchi through seeds on a commercial scale. New plants often come into fruit bearing after a prolonged vegetative phase (Hamilton and Yee 1970) and fruit quality is much inferior to their parental types (Joubert 1970; Loebel 1976). Nevertheless, seed propagation is vital for the development of new selections (Kadaman and Slor 1974) for root stocks and for breeding purposes.

As mentioned earlier, major problem associated with litchi propagation through seeds is their short life span and rapid loss of viability under desiccation (Menzel 1985; Pandey and Sharma 1989; Fu et al. 1990; Kumari-Singh and Prasad 1991). Litchi seeds may keep well within fruit up to a month but start losing viability even within a day after separation from the fruit (Menzel 1985; Prasad and Prasad 2004).Cull and Paxton (1982) earlier reported best storage of seed while inside the fruit or in moist peat moss in a freezer. Optimum seed germination was observed when seeds were sown immediately after their separation from the fruits (Prasad et al. 1996) and effect of desiccation on their germination was investigated by Xia et al. (1992a and b), Prasad and Prasad (2004).

7.13.1 Conventional Propagation of Litchi

Conventionally, litchi propagation involves asexual means to develop new plants true to the parental type (Syamal and Mishra 1984; Pandey and Sharma 1989; Menzel 1985). Air layering, grafting, budding and stolling are other means of litchi propagation. Of these, air layering is the most common and successful; it is called gootee in India. Grafting and budding are not in vogue because of non-availability of vigorously growing healthy seedlings for use as root stocks. The major shortcomings of marcottage and grafting in developing plants involved depletion of branches in large numbers from the mother trees besides being slow and inefficient (Chapman 1984). Therefore, alternative efficient breeding methods that can provide new plants true to the parental types in large numbers while overcoming the major shortcoming of asexual means of litchi propagation are the forms of research.

There is a great potential for *in vitro* regeneration of litchi. If successful, it may develop into a major industry. Selected elite cultivars need to be multiplied through cloning on a large scale evaluated at the field level and preserved on a long term basis (Sarin and Prasad 2003). Some attempts have been made in this regard although it is a difficult task considering initial difficulties in propagating through *in vitro* techniques (Kantharajah et al. 1989). However, callus induction, somatic embryogenesis and plant regeneration from immature zygotic embryos and anthers have been reported (Zhou et al. 1996; Kantharajal et al. 1992; Fu and Tang 1983).

Yu and Chen (1998) further reported development and maintenance of androgenic suspensions and protoplasts isolated from several litchi cultivars that was followed by somatic embryogenesis and plant regeneration from protoplasts isolated from zygote derived embryogenic suspensions of litchi var. xiafanzhi (Yu et al. 2000). Das et al. (1999) reported multiple shoot formation and plant regeneration from the cotyledonary nodes as well as by *in planta* treatment of the axillary bud regions. In another study, a reproducible method of in vitro regeneration of elite litchi trees for clonal propagation has recently been reported (Kumar et al. 2006).

7.13.2 In Vitro Regeneration (Micro-propagation) of Litchi

Litchi can be propagated through various asexual means such as grafting, stem cutting, air layering or marcottage and budding (Menzel and Simpson 1987; Pandey and Sharma 1989). These methods are useful in raising 'true-to-the parental type' plants and desired cultivars are properly maintained and become easily available in large numbers. However, such propagation methods involve depletion of branches in great numbers from the mother trees (Ray and Sharma 1985). Although conventional vegetative propagation methods are slow and inefficient (Chapman 1984), these methods are used under horticultural practices in many countries. Several researchers have used auxins indole butyric acid (IBA) and α-napthalene acetic acid (NAA) to promote root initiation and better root development. Genotype, physiological condition and wood type of the parent tree in addition to environmental factors are important considerations for successful development of a root system by stem cuttings (Pandey and Sharma 1989).

Grafting and budding are not in vogue primarily because of the non-availability of vigorously growing healthy seedlings of litchi for use as root-stocks. Therefore, alternative methods for raising the chosen elite cultivars must be attempted. Regeneration of litchi *in vitro* is an alternative to vegetative propagation for mass scale production of desired cultivars. This technique also spares branch depletion. *In vitro* regeneration of litchi that is a prerequisite for genetic engineering might also prove beneficial in improving the shelf life of fruits as well as enhancing the viability of seeds using appropriate genes and transformation methods.

Initial attempts towards clonal multiplication of litchi using seedlings and mature tissues failed to yield any positive results (Wolf 1987; Kantharajal et al. 1989). However, protocol for clonal propagation or direct regeneration through nodal cuttings has been established for further improvement (Kumar et al. 2006).

7.13.3 Direct Regeneration from Nodal Explants of Litchi

Micro-propagation is defined as the true-to-type propagation of selected genotypes using *in vitro* culture techniques. Depending on the species and cultural conditions, *in vitro* propagation can be achieved by the following three basic methods (Kane

1996): (a) Enhanced axillary shoot proliferation (shoot culture), (b) Nodal culture and (c) *De novo* adventitious shoot formation through shoot organogenesis.

Kantharajal et al. (1992) was the first to demonstrate a method for litchi regeneration through *in vitro* technique. This method involved the embryos of different sizes and ages from commercial varieties of litchi that were cultured in a range of different media. They reported that pre-treatment of embryos with 100 mg^{-1} BA in liquid medium for 3 hours was optimum for adventitious shoot formation. Subsequent transfer to MS semi-solid medium containing thiadizuran (1 mg l^{-1}) resulted in the formation of 5 shoots/explant in cv. Bengal. Das et al. (1999) suggested two methods of shoot multiplication. The first involved shoot bud initiation from seeds directly germinated on filter-paper bridge submerged in liquid MS medium supplemented with 20 mgl^{-1} BA. The second method (*in planta*) involved the use of 4–5 week old seedlings that had been germinated and grown on vermiculite in culture bottles. The leaf axils (nodal region) of these seedlings were treated with 100 μl solution of various concentrations of BAP ranging from 0.25 to 1.0 mg/ml that was supplemented on alternate days through a moist filter paper placed in direct contact with axillary meristem for 8 weeks. Highest number (8) of multiple shoots was observed after 7–8 weeks of BAP (1 mg/ml) treatment. Both methods of multiple shoot induction were effective for the five genotypes of litchi Chandra and Padaria (1999) cultured shoot buds of litchi cv. Seedless on MS medium supplemented with 0.2 mg BA + 0.1 mg IAA + 0.5 mg GA_3/litre and obtained shoot differentiation and growth.

Kumar et al. (2006) established for the first time a rapid regeneration system through *in vitro* culture of litchi by culturing nodal segments obtained from field grown plants. Different cytokinins were tested; explants grown in the presence of BAP, 2-iP, Kin and other additives (coconut water, casein hydrolysate, silver nitrate etc.) gave rise to multiple shoot formation from the nodal segments.

The cytokinins were effective only when provided in moderate concentrations over a period of time, while higher concentrations proved counter-productive. BAP at a concentration of 11 μM was most suitable for the regeneration from nodal explants and 6 μM BAP was the optimum for further elongation and multiplication of shoots.

Pulse treatment of IBA to the well developed shoots followed by culture in MS medium supplemented with IBA (20 μM) and litchi seed powder (1 g/l) was found to be the best rooting medium for litchi. *In vitro* grown plantlets were successfully transferred to the field and they are surviving in the harsh climatic condition of Delhi. This system is ideally suited for mass scale propagation and may also be amenable for further industrial application.

7.13.4 Somatic Embryogenesis

Somatic embryogenesis is the process by which somatic cells develop through the stages of embryogenesis to give whole plants without the fusion of gametes. Somatic embryogenesis was defined by Emons (1994) as the development from somatic cells of structures that follow a histodifferentiation pattern that leads to a body pattern

resembling that of zygotic embryos. *In vitro* somatic embryogenesis can either occur directly from callus or suspension culture (Williams and Maheswaran 1986).

7.13.5 Induction of Embryogenic Callus and Histological Study

Somatic embryogenesis has been attempted in litchi. Zhou et al. (1996) reported that MS medium supplemented with 2.0 mgl^{-1}2, 4-D + 0.2 mgl^{-1} BA + 0.12 mgl^{-1} NAA was ideal for embryogenic callus formation from immature embryos (20-, 30- and 50-day-old) of 4 varieties of litchi, although rapidly growing calli were also obtained on MS medium containing 4.0 or 6.0 mgl^{-1} 2, 4-D + 0.1 mgl^{-1} BA + 0.1 mgl^{-1} NAA. Somatic embryo development and complete plantlets were obtained on MS medium supplemented with low concentration of NAA and IBA. Yu and Chen (1998) reported the induction of litchi (Xiafanzhi) embryonic calli from immature embryos and anthers cultured in vitro. The immature zygotic embryos were removed and transferred onto induction medium (MS1) that included MS salts B_5 vitamins, 50 gl^{-1} sucrose and 2 mgl^{-1} 2, 4-D. The cultures were maintained in darkness and the embryogenic callus that appeared after 6–8 weeks of culture was pale yellowish and friable. Yu and Chen (1998) utilised this callus to generate embryogenic suspension culture. Liao and Ma (1998) carried out a thorough investigation of somatic embryogenesis and plantlet regeneration in litchi *cv*. Yuherbau. Secondary embryos appeared in large numbers on the surface of immature primary somatic embryos in a culture medium containing 0.05 mg/l NAA, 0.05 mg/l 2-ip, 0.2 mg/l ABA. According to several researchers (Merkle 1995; Yu et al. 2000), culturing on medium supplemented with 2, 4-D followed by callus growth onto the medium devoid of 2, 4-D gave rise to somatic embryos and eventually to the plantlets. Histological studies of somatic embryos of different species have been described for both pathways of origin: unicellular and multicellular (Vasil and Vasil 1982; Alemanno et al. 1996).

7.14 Conclusions

Mutagenesis approach would be ideal for developing new mutant lines and also for enhancing germplasm of litchi. International Atomic energy Agency (IAEA) maintains mutant variety database (www.iaea.org) that includes over 2,600 officially released mutant varieties of various crops in different countries. A wide range of mutants would be of great use in molecular characterisation and identify trait specific molecular markers. These markers will be helpful in molecular marker assisted selection and breeding.

Plant regeneration of litchi through *in vitro* techniques on a mass scale would require more research as this area is still in its infancy. Vegetative propagation of litchi maintains 'true-to-type' nature of cultivars as propagation through seed would produce inferior cultivars in terms of fruit quality, size and maturity period. How-

ever, diversification of litchi cultivation also demands other characters like precocities, dwarfness, regularity in bearing wide adaptability and resistance to disorders need to be considered. Asexual means of propagation cannot provide variations needed for such purposes whereas sexual means produces a large number of variants. Thus there is an urgent need for raising plants through seeds because now variants would provide better selections.

In the last 20 years breeding programmes in litchi had been undertaken on a large scale. For instance, South Africa aims at 100 seedlings for evaluation and selections annually. In China, breeding and selection for extension of bearing period and quality improvement have resulted in four new selections including 'Aili', a dwarf selection and 'Ziangxi' with large fruits (39.1–59.5gm). In Australia, genetic improvement through reciprocal controlled breeding among several existing cultivars gave rise to new seedlings. With shortened vegetative phase, fruit production starts within 3 years of plantation.

Breeding for selection of new cultivars has immense potential and holds a promising future in tackling problems of browning, prolongation of self-size and extension of the breeding period of the litchi.

Further advent of new technologies involving DNA-based molecular markers like 'RAPD' and 'AFLP' are quite useful in assessment of genetic diversity, benefiting the breeders in parent selection and new combinations of characters in their progeny.

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Chapter 8
Avocado Genetics and Breeding

E. Lahav and U. Lavi

8.1 Introduction

Avocado is highly heterozygous resulting in unpredictable progeny. Single gene mutations are unknown except for DNA markers. Avocado has only one seed per fruit and is characterised by heavy fruit drop, a long juvenile phase and a large tree size resulting in a substantial area required for a reliable assessment of hybrids. The advantages for the breeder are the wide genetic variation and the ease to vegetatively propagate the selected seedlings.

Avocado breeding programmes have been reported in California (Lammerts 1942, 1945; Schroeder 1960; Bergh 1961), Australia (Sedgley and Alexander 1983), South Africa (Du Plooy et al. 1992; Bijzet and Cilliers 1995), Mexico (Sánchez-Colin and De la Cruz-Torres 1992) and Israel (Lavi et al. 1991b). General reviews on avocado breeding were published by Bergh (1969), Bergh and Lahav (1996) and Lahav and Lavi (2002). The following chapter is based in part on the last two reviews.

8.2 A Short Taxonomic Description

Commercial avocado (*Persea americana* Mill.) belongs to the sub-genus *Persea* that also contains two other species, *P. schiedeana* (Nees) and *P. parviflora* (Williams). *P. americana* is a polymorphic species containing several separate taxa that are considered to be botanical varieties more commonly referred to as horticultural races (Scora et al. 2002). Botanical varieties that lie within *P. americana* include *P. americana* var. *drymifolia*, *P. americana* var. *guatemalensis* and *P. americana* var. *americana* (Bergh and Ellstrand 1986). These are commonly known as the Mexican, Guatemalan and West Indian (Lowland or Antillean) horticultural races

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respectively, based on their presumed centres of origin (Popenoe 1941). The number of chromosomes in avocado is $2n = 24$ (Garcia 1975).

There are no sterility barriers among the three races or among any taxa classified under *P. americana*. Hence, hybridisation occurs wherever trees of different races are growing in proximity, whether indigenously (Popenoe and Williams 1947) or under cultivation (Bergh 1969). 'Fuerte', the cultivar that long led production in California and most other Mediterranean/subtropical growing regions, is apparently a natural Mexican × Guatemalan hybrid, although with predominantly Mexican race characteristics. 'Hass', currently the dominant cultivar in most of these regions, is generally regarded as pure Guatemalan, but progeny produced by self-pollination indicate that it contains Mexican genes (Bergh and Whitsell 1974). Guatemalan × West Indian hybrids are currently the leading cultivars in Florida and look promising for future production in more tropical areas. For a detailed description of avocado taxonomy, see Scora et al. (2002).

8.3 History of Improvement

Two steps are usually involved in fruit tree improvement: selection of improved genotypes and their fixation by vegetative propagation. Avocado produces only sexual seeds and flowering dichogamy somewhat favours cross-pollination. Seedlings produced by a single tree (or cultivar) are extremely variable and in most instances have a prolonged juvenile period. The few selected seedlings, resulting from breeding projects that produce high yield of high fruit quality, must be vegetatively propagated as their sexual progeny have significant variation in fruit and tree characteristics. The first known grafting of avocado took place in Florida before 1900 (Ruehle 1963).

Selection of horticultural improved avocados occurred long before they were asexually propagated. Avocado seeds of varying antiquity (beginning about 7000 B.C.), excavated in Mexico (Smith 1966), indicated that selection for large fruit could have begun about 4000 B.C. However, this conclusion was essentially based on a comparison of the largest seeds at each level of the excavation. The number of seeds present was greater in the later deposits and sample size was strongly correlated with the largest individual seed size. Thus, Smith's data are compatible with selection for larger avocado fruits over the past several thousands years despite the absence of conclusive evidence. In addition, it seems reasonable that selection would also have occurred for a smaller proportional seed size as well as a larger fruit size (Popenoe 1919). However, there are no data to support this conclusion.

Extensive pre-Columbian avocado selection is evident from the high horticultural quality already present when Europeans first encountered the avocado. This was likely to have occurred through the laborious process of selection and propagation of superior cultivars by seed from small-fruited wild forms found in the forests of Mexico and Central America. Popenoe (1919) suggested selection methods as

follows: cutting down the poorer seedlings, planting seeds from superior seedlings, selling choice fruit and thereby spreading the better types. It has been suggested that the smaller Mexican fruit size indicates less selection within that race (Chandler 1958). Further selection has occurred during the last century with superior cultivars being preserved through vegetative propagation (Popenoe 1952).

The selection procedure in avocado is illustrated by the Rodiles orchards near Atlixco (Mexico) where a seedling population is said to be non-variable (Anderson 1950). For generations, the Rodiles family planted seeds from the finest available local avocados, resulting in thousands of fruit bearing trees with high quality. 'Fuerte', long the world's leading cultivar, was developed in this orchard as was 'Puebla' that also was a leading California cultivar for many years (Kellogg 1971). In contrast, the authors of this chapter have always encountered great variation among seedling populations (Lavi et al. 1993b). A most zealous programme to improve the avocado industry by introducing scions of superior seedlings growing in indigenous habitats was centred in Guatemala and culminated in 1917 with the introduction to California of 'Nabal', 'Benik' and several other Guatemalan race cultivars. The marketing results were regarded as disappointing as at that time it was not realised that the preference of Californian consumers was for a much smaller fruit than is preferred in the tropics. Most of the introductions produced large fruit while others cropped poorly under California conditions or lacked other desirable characters. Nevertheless, some of these introductions made genetic contributions to the development of present day cultivars. Popenoe (1951) selected a few additional seedlings from the Rodiles orchard (predominantly Mexican types), looking for a wide germplasm for Central American avocado production. In addition, sporadic introductions of seeds from superior types in Central America and in adjoining regions widened the genetic background of commercial cultivars in California, Florida and elsewhere (Bergh 1957). All current important Florida cultivars were selected from locally grown seedlings produced from open-pollination. These were predominantly West Indian and (more recently) West Indian × Guatemalan hybrids. Several tropical areas have also advanced their regional avocado industries by selecting and vegetatively propagating superior local seedlings. All major current commercial cultivars in California (with the exception of 'Fuerte') are local random selections.

8.4 Breeding Objectives

We distinguish between "Specific" and "General" objectives. The first refer to situations where the breeder has a specific objective such as introducing a specific resistance gene like resistance to fire-blight in pears or seedlessness in citrus. Such objectives are usually found in the breeding of well-established crops where good cultivars already exist and there is a need to improve them. "General" objectives are those characterised breeding of "exotic crops" where the breeders and growers

are interested in cultivars that are better than the current available ones but have no major restrictions regarding the fruit characteristics of the new cultivars.

Avocado breeders have 'General' breeding objectives and are interested in high quality fruit with long shelf life and high yield and are not restricted to a specific colour, shape and size in which producer and/or consumer preferences change with time (however, the American breeders prefer the 'Hass' colour and shape). The following are the current mainstream objectives:

8.4.1 Rootstocks

8.4.1.1 Phytophthora Root Rot

Avocado rootstock selection and breeding was reviewed by Ben-Ya'acov and Michelson (1995). Adequate resistance to *Phytophthora cinnamomi*, which causes root rot, is the most desired trait throughout the avocado world. Extensive attempts to hybridise avocado with *Persea* species of sub-genus *Eriodaphne* that are resistant to the disease have failed. An alternative approach was suggested by Witjaksono (1997) and by Witjaksono and Litz (1998) who developed protoplast fusion and somatic hybridisation methodology to assist with the provision of *Phytophthora cinnamomi* resistance and salinity tolerance in avocado. The last available report on this approach is Litz (2005). Thus, through this technology sexual and graft incompatible species as *P. borbonia*, *P. caerulea* and *Machilus* spp. may be hybridised with *P. americana* (Pliego-Alfaro et al. 2002). The assumption of this approach is that cytoplasmic genes control the resistance. At this stage, regeneration of somatic hybrids is the limiting factor. Limited resistance to Phytophthora root rot is known in certain lines of avocado and the closely related *P. schiedeana*. A few selections from them are important, especially in California, and have been clonally propagated to maintain the resistance level. 'Duke' and its derivatives ('Duke 7', 'Barr-Duke' and 'D9') and 'Thomas' are the most important ones (Newett et al. 2002; Pegg et al. 2002). For decades, Zentmyer (1972) and his successors have been searching in Mexico and Central America for wild sources of root rot resistance. The Mexican-race 'G6' achieved some commercial use. Another import, 'Martin Grande' (G755), evidently a cross between *P. schiedeana* and a Guatemalan-race tree (Ellstrand et al. 1986), has a comparatively high level of resistance but usually produces low-yielding trees due to excessive vigour.

The South African random seedling 'Dusa', of Guatemalan-Mexican origin, is reported (both in South Africa and California) to be significantly more root rot tolerant and more productive than 'Duke 7' (Kremer-Kohne and Mukhumo 2003).

Zentmyer et al. (1965) reported Guatemalan rootstocks to be more sensitive than Mexican rootstocks to *Dothiorella* and *Verticillium* wilt. Ben-Ya'acov and Frenkel (1974) found significant differences in sensitivity to *Verticillium* wilt among different West Indian rootstocks, while Tsao et al. (1992) found that some rootstocks tolerant to *P. cinnamomi* are sensitive to *P. citricola*, a serious pathogen of the avocado. No resistance to *Dematophora necatrix* is known.

8.4.1.2 Salinity

Resistance to salinity is greatest in the West Indian race and least in the Mexican race cultivars. However, there is significant variability within each of the three races (Kadman and Ben-Ya'acov 1976) and even among seedlings from the same tree (Kadman 1968). Resistance to high-lime chlorosis is greatest in West Indian cultivars and there is also a considerable intra-racial variability in this trait (Ben-Ya'acov 1972). However, West Indian rootstocks perform poorly in heavy soils and under waterlogged conditions (Ben-Ya'acov et al. 1974). A major Israeli objective is to combine the West Indian tolerance to high salinity and lime with tolerance to low oxygen. Breeding for rootstock tolerance to salinity and chlorosis is also reported from Mexico (Sánchez-Colin and Barrientos-Priego 1987) and South Africa. The South African selection 'Dusa' showed in California a good measure of tolerance to saline irrigation water (Crowley and Arpaia 2002).

Pure West Indian rootstocks have not proven satisfactory in the winter-cold California soils, but hybrids especially with Mexican genes seem well adapted. A rootstock that enhances scion cold hardiness would be highly desirable in frost-prone areas, but chill-tolerant Mexican rootstocks that have been studied did not transmit their hardiness to the scion (Halma and Smoyer 1951; Ben-Ya'acov 1987, 1998).

8.4.1.3 Dwarfing

Sánchez-Colin and Barrientos-Priego (1987), reported a significant dwarfing effect by their 'Colin V-33' selection, whether used as an inter-stock or as an ordinary seedling rootstock. However, since viroid was identified in some tests of this rootstock, its use for dwarfing is doubtful. A dwarfing rootstock could be a major benefit for most avocado producers. In Israel, the West Indian cultivars of 'Nahlat' (Ben-Ya'acov et al. 1979) and 'Maoz' (Kadman and Ben-Ya'acov 1980) were found to have dwarfing effects. In other studies, trees grafted to Mexican rootstocks were smaller than comparable trees grafted to West Indian types (Ben-Ya'acov 1976).

8.4.2 Cultivars

8.4.2.1 Fruit Traits

Size – The present optimum fruit size for most markets is about 250–350 g. For the sophisticated markets of developed countries fruit outside the 170–400 g range is unacceptable. Size is the most variable trait for a given genotype, being affected by crop load, proximity to other fruit on the tree, stage of maturity, cultural practices, climatic conditions and other unknown factors (Lahav and Kalmar 1977; Whiley and Schaffer 1994). Bergh and Lahav (1996) reported that in every selfed progeny set, the average size of seedling fruit was smaller than the parent, but this phenomenon was not repeated in the Israeli breeding project.

Shape – Fruit shape segregates extensively in most self-progenies. The squat-pyriform shape of 'Hass', the ovate fruit of 'Bacon' and the thick-ovate form of 'Gwen' are all desirable shapes. Fruits that are too elongate are quite common in the progeny of 'Hass' (Bergh and Whitsell 1974). The excessive elongated shape of 'Pinkerton' or 'Galil' (selected in the Israeli breeding project) can be shortened by treatments with growth retardants (Lahav et al. 1998).

Skin thickness – The leathery easy-peeling type of 'Fuerte' or the thicker skin of 'Hass' (for spoon eating) are usually preferred. Thin-skinned fruit is prone to damage while a skin that is too thick prevents determination of ripening time.

Skin colour – The preferred colour varies with market and time. The black-skinned 'Hass' was down graded during the time when green skinned 'Fuerte' was the leading cultivar in the US. Now that 'Hass' is dominant, cultivars with green skins sell for less. Breeders should probably ignore such passing fashions and, in our opinion, should concentrate on quality traits. However, currently, breeding for black skins, similar to 'Hass' is a major objective worldwide. Fruit that have mixed green and purple skins (seen in a considerable proportion of selfed 'Nabal' progeny) are usually less attractive. Glossy skin surfaces as characterised by 'Ettinger' are commonly considered attractive. In California, the severe russeting of 'Regina' and the end-spotting of 'Zutano' are unfortunately present in a majority of their respective progeny.

Seed size – Variability in seed size is very common within the same progeny (Fig. 8.1). A small seed relative to fruit size that is tight in the pulp cavity is a superior attribute of many Guatemalan lines. 'Irving' (whose later-maturing fruit and slightly thicker skin indicate more Guatemalan genes) has an exceptionally low seed/flesh ratio. The West Indian 'Ruehle' has small seed for that race. Elongated

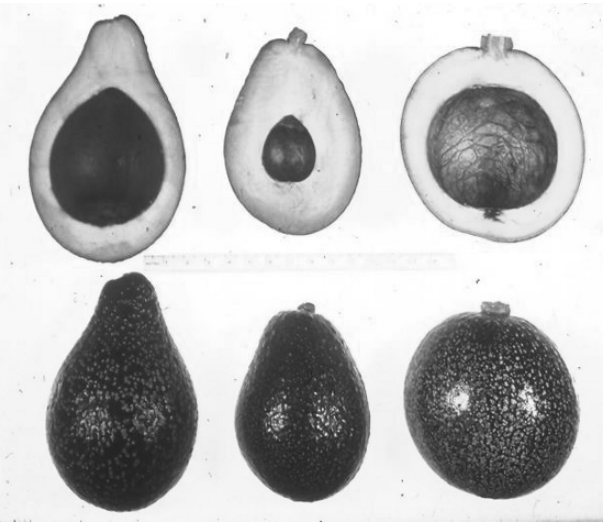

Fig. 8.1 Variation in seed size and fruit shape within seeding of the same progeny

fruits generally have a smaller seed with the long-necked ‘Pinkerton’ epitomising this trait. The semi-commercial ‘H287’ (a progeny from the Californian breeding project) has similarly a very small seed in a better shaped fruit. Progenies of both on average have smaller seeds than most other seedling groups. It is highly desirable that both seed coats remain attached to the embryo instead of to the flesh (loose seeds are frequently found in Mexican types).

Ripening – In most cultivars, the fibrovascular bundles tend to become more prominent with increasing maturity, but this undesirable trait is less marked in most Guatemalan lines. Uniform and adequate softening of fruit as it ripens is independent of race. For example, ‘Jalna’ (Mexican) and ‘Pinkerton’ (Guatemalan hybrid) are characterised with uneven ripening that may be more pronounced in some regions or in a fruit with advanced maturity (Piccone and Whiley 1986). A long period from harvest to softening is advantageous, especially when fruit is transported long distances. However, the unusually slow-ripening ‘Pinkerton’ has caused some consumer dissatisfaction because of its delayed edibility as compared with other cultivars. A longer time from softening to flesh deterioration is highly desirable. Avocado has a short shelf life compared with other fruits, although it varies between very short in ‘Anaheim’ to the better-keeping qualities of ‘Hass’, ‘Fuerte’ and others. West Indian lines have much lower oil content than those of the other two races but there is considerable intra-race variability. Guatemalans and especially Mexicans can reach well over 20% oil, but the ‘Anaheim’ and ‘Mayo’ cultivars of these two respective races may have an oil content below 8% when they begin dropping or deteriorating.

Flavour – The rich, slightly nutty taste of ‘Hass’, ‘Fuerte’ or ‘Benik’ is generally preferred over bland flavours. However, population preferences do exist and while the spicy or anise-like flavour of Mexican types such as ‘Mexicola’ or ‘Duke’ is more desirable by some consumers, the mild, sweeter taste typical of West Indian cultivars is usually preferred by other populations (such as in Central America).

Fruit diseases – In high rainfall climates, where fruit diseases are common, genetic resistance is desirable since fungicide treatments are expensive, not fully effective and are unfriendly to the environment. Ruehle (1963) lists the following relative cultivar susceptibilities: Cercospora spot or blotch (caused by *Pseudocercospora purpurea* Cooke) is much less severe on ‘Collinson’, ‘Fuchsia’ and ‘Pollock’; ‘Fuerte’ and other Mexican types are highly susceptible to anthracnose or black spot (caused by *Colletotrichum gloeosporioides* Penz.); ‘Fuchsia’, ‘Pollock’, ‘Booth 1’ and ‘Waldin’ are quite resistant while ‘Lula’ is highly susceptible to avocado scab (caused by *Sphaceloma perseae* Jenkins).

8.4.2.2 Tree Characteristics

Yield – The most important tree characteristics are precocity with consistent and high yields. Without this, excellence in the other tree characteristics is meaningless. Production consistency from year to year may be as important as overall heavy production (Bergh 1961). Because of large differences in tree size, yield is best

assessed as tonnes per hectare at the respective tree spacings. A more subjective evaluation is fruit number per tree volume or "covering area" of the tree canopy (Kadman et al. 1976). The majority of fruit should reach commercial maturity about the same time and this is especially important for earlier-season cultivars that have a short life on the tree. Nothing is known about the heritability of this trait that is assessed in the second selection stage (see below).

It is noteworthy that there are reports describing a significant increase in yield as a result of treatments with various growth regulators (Wolstenholme et al. 1990). We believe that increasing the yield by selection of high yielders is preferable in the long run but at the same time, breeders should be aware of developments in horticultural practices and focus (in the short run) on those goals that are unachievable by agrotechnical procedures.

Architecture – Tree form varies from erect as typified by 'Reed' through spreading ('Fuerte') to weeping as in 'Wurtz'. A spread about equal to tree height is considered desirable and dwarf or semi-dwarf erect trees are considered ideal. Very tall trees such as 'Bacon' or 'Ettinger' are difficult to manage and make fruit harvest expensive. Excessive vigour and fruitfulness are not fully compatible and exceptionally robust seedlings usually have little or no fruit. According to Bergh et al. (1996), tree vigour declines with inbreeding and is restored when inbred lines are crossed. Enough vigour to maintain good tree health and high productivity is essential.

Cold tolerance – Most of the major world avocado regions are subject to occasional frost damage and cold tolerance is highly advantageous for both the fruit and tree as a whole. Outstanding cold hardiness is limited to the Mexican race while above-freezing temperatures may still injure West Indian race cultivars. 'Hass' has been considered an extraordinary cold-hardy Guatemalan cultivar, but its progeny suggest that perhaps a fifth of its genes came from the Mexican race (Bergh and Whitsell 1974). Mhameed et al. (1997) and Davis et al. (1998) also provide evidence for an inter-racial origin for 'Hass'. This presumably explains both its cold tolerance and the fact that it is palatable much earlier in the season than pure Guatemalan cultivars. There are differences in tolerances within as well as between races (Fig. 8.2). For example, among cultivars believed to be pure Guatemalan, 'Nabal' and 'Reed' are unusually cold hardy and 'Anaheim' unusually sensitive to low temperatures. 'Yama' is considered one of the hardiest Mexican cultivars withstanding $-8\,^{\circ}C$ without severe injury, which would make it a favourable parent for cold hardiness breeding. Mexican seedlings appear promising in Florida for generating commercial selections with enhanced cold hardiness to withstand the freeze conditions that periodically occur there (Knight 1971).

Heat tolerance – Heat tolerance of cultivars varies significantly. However, the Mexican race has greater average heat tolerance, as exemplified by 'Mexicola', 'Mayo' and 'Indio'. Moreover, because Mexicans bloom earlier, their fruits are usually more advanced and thus less vulnerable to spring heat waves. 'Frey' and 'Hass' appear to be among the more heat-sensitive Guatemalan cultivars. 'Irving', a Mexican-Guatemalan hybrid, has shown exceptional tolerance to desert heat and low humidity in California (Bergh et al. 1996).

Fig. 8.2 Variation in cold hardiness within seedlings of the same progeny

8.5 Floral Biology

The flowers are grouped in terminal, highly compound cymes or thyrses (Scora et al. 2002) of dozens to hundreds of flowers each. Hence, a single tree may have a million flowers during one blooming period and only 100–200 fruits (Lahav and Zamet 1999). This low rate of fruit set makes hand-pollination impractical.

Knowledge of avocado floral biology is important for the generation of both selfed and crossed progeny. Avocado flowering has been reviewed by Bergh (1986), Davenport (1986) and by Gazit and Degani (2002). The avocado flower is protogynous, i.e. its pistil is receptive before pollen shedding. The flower opens twice for several hours each time and each opening is separated by at least one overnight period. The flower is functionally pistillate (female) during the first opening and staminate (male – pollen shedding) during the second opening. Under warm temperatures, avocado cultivars fall into one of two flowering groups (Lahav and Gazit 1994):

Group A – the first (female) opening starts in the morning and ends before noon. Second (male) opening occurs in the afternoon of the next day.
Group B – the reverse pattern: the female opening occurs in the afternoon and male opening the next morning.

Thus, the two groups are reciprocal, favouring cross-pollination, although self-pollination and pollination within trees of the same flowering group is quite frequent (Degani and Gazit 1984). As the weather becomes cooler, the opening and closing of the flowers are delayed. In addition, more than one night may elapse between the first and second openings (Gazit and Degani 2002).

Length of flowering varies with cultivar and climate – the cooler the temperature, the longer the flowering period. Guatemalan cultivars usually bloom later than those of the other two races. Usually, each tree flowers continuously for about two months and it is rare for the earliest to be finished before the latest begins. The breeder

can manipulate flowering time to achieve overlap of desired parents. For example, flowering can be advanced as much as 2–3 months by girdling (Lahav et al. 1972) or by placing container-grown trees in a greenhouse during winter. Similarly, flowering can be delayed by holding such trees in a cool growth chamber or scions with differentiated flower buds can be stored at about 4 °C and grafted at the breeder's convenience. Using the latter technique, it is possible to carry out pollination for six months (Sedgley and Alexander 1983). Growth retardants have had limited success on controlling the flowering period (Levin 1981).

8.6 Genetics of the Juvenile Phase

The length of the juvenile phase is a major factor affecting the efficiency of fruit tree breeding programmes in general and avocado in particular. Relative to other fruit trees, this period in avocado is quite long reaching 15 years or more (Bergh et al. 1996). The efficiency of genetic improvement is inversely related to the length of the breeding cycle. Thus, it is important to shorten the juvenility phase. In breeding programmes based on controlled crosses, it may take about 18 years before a selection is released for small-scale commercial evaluation (Table 8.1). In breeding based on open-pollination and a fast release of selected progeny, the cycle may be reduced to about 11 years.

The length of the juvenile period is genetically controlled as shown in some fruits (Johnson 1940; Visser et al. 1976). The juvenile period in avocado can be significantly shortened by choice of parents (Lavi et al. 1992). 'Pinkerton' and 'Gwen' (Bergh et al. 1996) and 'Arad' (Lahav et al. 2005) produce very precocious

Table 8.1 Approximate timetable for avocado breeding

Year	Stage	Controlled Crosses	Year	Open Pollination
1	I	Preparation of parents	1	Pollination
2		Continued preparation of parents	2	Nursery
3		Breeding in cages	3	Planting in field
4		Nursery germination	4	Continued planting in field
5		Planting in field	5	Girdling
6			6	Evaluation and selection
7		Girdling, if practiced		
8		Evaluation and selections		
9		"		
10		"		
11		"		
12	II	Nursery propagation.	7	Top-working
		Top-working (may begin sooner)	8	Intense evaluation
			9	"
13		Field planting	10	"
14		Intense evaluation	11	"
15		"		
16		"		

offspring, sometimes fruiting two years from grafting. In contrast, Lavi et al. (1992) reported that the mean flowering age ranged between 4.3 years for the progeny of 'Rosh-Hanikra II' x 'Ettinger' to 8.6 years for the self-pollinating progeny of 'Nabal' (Table 8.2). In their study with 11 progeny populations, flowering age ranged between 3 and 11 years (with some seedlings up to 14 years). Fruiting age ranged between 3 and 11 years (up to 14 years in one case). No seedling flowered or fruited earlier than 3 years after planting and only 4.2% did so at the age of 3 years (Table 8.2). Note that a significant portion of each progeny population did not flower or fruit during the 10 years of experimentation. Statistical analysis showed clearly that the various progeny populations differ significantly in their flowering and fruiting ages. A difference by a factor of two between the earliest and the latest mean flowering age probably depends on genetic factors that are quantitatively controlled. Although there were some variations between different years, the above mentioned figures represent the general picture. No differences were detected between self- and cross-pollinations suggesting that there is no significant effect of heterozygosity level on the juvenile period (Lavi et al. 1992). It is noteworthy that the majority of seedlings that flowered in a certain year, fruited the same year or the following year, suggesting that the main constraint in an avocado breeding programme is time until the first flowering. It was concluded that the choice of parents might influence the length of the juvenile period and thus the efficiency of the breeding project. However, choice of parents in breeding programmes is influenced by other factors as well and thus the use of this tool is quite limited.

Table 8.2 Length of the juvenile period in 11 avocado progeny populations

		Flowering Age (Years)				Fruiting Age (Years)			
Cross[z]	Number of Progeny	*Mean*	*SD*	*Range*	*(%) flowered*	*Mean*	*SD*	*Range*	*(%) fruited*
Rosh Hanikra II × Ettinger	51	4.3[y]	0.7	3–7	90.2	5.4	1.0	3–7	62.7
Tova × Fuerte	48	5.0	1.5	3–14	91.8	5.6	2.0	4–14	37.7
Hass × Fuerte	123	5.8	1.7	3–10	86.1	6.3	1.9	4–11	70.8
Ettinger (selfed)	235	5.9	1.1	4–8	70.7	6.9	0.8	5–8	42.1
Ettinger × Tova	62	6.2	1.7	3–10	91.9	7.0	2.0	4–10	83.9
Tova × Regina	54	6.2	0.9	5–7	88.9	6.5	0.8	5–7	79.6
Hass × Ettinger	46	6.4	1.3	4–8	93.6	6.9	1.2	5–8	89.2
Anaheim (selfed)	61	6.5	0.6	5–7	68.9	6.5	0.7	5–7	32.8
Tova × Ettinger	387	7.0	2.1	3–11	77.4	7.0	1.9	3–10	37.7
Horshim × Tova	240	7.5	1.9	4–10	53.3	7.5	1.3	5–9	19.2
Nabal (selfed)	85	8.6	1.5	6–10	40.1	9.4	1.0	7–10	29.4

From Lavi et al. (1992), with permission.

[y] Order is from low to high mean of flowering age, based only on seedling that flowered and yielded during the experiment.

[z] Data refer only to the seedlings that flowered and fruited during the experiment.

8.7 Breeding Techniques

8.7.1 Shortening the Juvenile Period

Several horticultural practices have been applied to shorten the juvenile period. Top working has been used on a limited scale in Australia (Sedgley and Alexander 1983), Mexico (Sánchez-Colin and Barrientos-Priego 1987), California and Israel. Alternative approaches are to cut off a tree or discarded seedling and graft one new seedling on each or graft up to 50 scions of different lines into branches of a large tree. This can be efficiently done on whole rows of trees cut down a year ahead of time to produce numerous upright shoots. The multi-graft approach reduces space but requires much more labour, including ongoing pruning to protect less-vigorous shoots and has the risk of confused identities. Both approaches, but in particular the multi-grafting technique, have the added risk of contaminating new seedlings with viroid from the recipient mother tree. Grafting on West Indian rootstocks is always recommended under saline conditions. In our experience top working did not affect the length of the juvenile period.

Attempts have been made in California and Israel to induce earlier fruiting by bending seedling branches into a horizontal position, but no appreciable gain was achieved. A shorter juvenile period is reported in Mexico by training the seedlings as a single stem then allowing them to branch when 2 m high (Barrientos-Priego et al. 1991). Breeders in Israel and Mexico have noticed that larger planting distances shorten the juvenile period, probably due to better illumination.

Growth retardants offer theoretical advantages for enhanced fruitfulness by shifting vegetative/reproductive competition to favour flowering/fruit set but may introduce undesirable distortions. In Israel, no effect on the juvenile period was observed after application of various growth retardants (unpublished data).

Girdling is probably the best way to shorten the juvenile period (Lahav et al. 1986). The earliest autumn girdling increased the proportion of seedlings that flowered from 47% to about 100% that nearly tripled flowering intensity (from rating 1.0–2.7) and significantly increased the proportion of seedlings setting fruit (14.9% vs. 65.4%). Most importantly, this resulted in a 7-fold increase in the number of fruits per tree (1.3 vs. 9.3) (Table 8.3). Note that in breeding programmes, nine fruits per tree permit a reliable evaluation of the seedling. In conclusion, the recommended practice to shorten the juvenile period available to the breeder are choice of parents (without reducing genetic variation) and girdling.

8.7.2 Pollination

Commercial avocado production requires pollen transfer by large flying insects, primarily honeybees in subtropical regions (Vithanage 1990). Pollen requires about 2 h to grow from the stigma to the ovary, depending on the prevailing temperature and about 48 h for sperm-egg union (Sedgley 1979). Pollen tube progress can easily be

Table 8.3 Effect of girdling date on flowering and fruit set of avocado seedlings[z]

Date of Girdling	Number of Seedlings	(%) Flowered	Flowering Intensity[y]	(%) Fruited	Average Number of Fruit per Seedling
12 Sept. 1983	73	99.2[a]	2.7[a]	65.4[a]	9.3[a]
18 Oct. 1983	64	93.7[a]	2.3[b]	54.8[ab]	6.3[ab]
22 Nov. 1983	43	90.9[ab]	2.0[b]	42.6[ab]	4.7[bc]
5 Jan. 1984	27	61.4[b]	1.0[c]	27.4[bc]	1.7[bc]
Ungirdled control	74	47.3[b]	1.0[c]	14.9[c]	1.3[c]

From Lahav et al. (1986), with permission.
Values within columns followed by different letters are significantly different.
Means were separated by Tukey-Kramer test ($P = 0.05$).

[z] All values are least-square means.
[y] Flowering intensity ranked from 1 = very little to 5 = profuse.

followed by fluorescence microscopy after staining with aniline blue (Tomer and Gottreich 1975).

Hand-pollination is feasible for producing a few fruits but cannot be applied for breeding projects where large amounts of seedlings are needed. Hand-pollination is carried out by picking male-stage flowers with dehisced anthers and daubing stigmas of female-stage flowers. For controlled self-pollination, male-stage flowers can be harvested, placed in agar and stored at 4 °C until female-stage flowers open. The pollen from each sac usually sticks in a clump to the opened valve until it is removed by insects or drops with the flower. Methods of pollen collection, such as vacuum devices, have not worked well with avocado. Avocado pollen has remained viable for up to 6 days under field conditions averaging about 27 °C and 60% relative humidity (Papademetriou 1974). Storage viability can be extended by reducing both temperature and humidity. Thus, Sedgley (1981) successfully stored pollen for 1 month at 4 °C and 1–23% relative humidity and for one year at −196 °C and 0% relative humidity in liquid nitrogen. This procedure permits crosses between a wide range of avocado cultivars with various flowering times. However, due to the gradual loss of pollen viability over time, it is advisable to use fresh pollen where feasible. Net caged unpollinated trees have set only few fruits in California (Peterson 1955) or Israel (Gazit 1977).

8.7.2.1 Controlled Pollination

The advantage of controlled pollination is the knowledge of parents' identity while the cost per seedling is much higher. Self-pollinated seedlings can be obtained by three basic procedures:

1. Fruit can be harvested from a source at a sufficient distance from any other avocado trees. Note that some cross-pollination by bees (or other insects) can occur over long distances (Torres and Bergh 1978; Vrecenar-Gadus and Ellstrand 1985; Degani et al. 1989). The further away from contaminating pollen, the greater the likelihood of selfing.

Fig. 8.3 Breeding cages with two cultivars of complimentary flowering groups enclosed in each age

2. The tree can be caged within a bee-proof net in the presence of a hive (Fig. 8.3). Sometimes high levels of cross-pollination have been detected probably due to wind-pollination (Degani et al. 2003).
3. Seedlings can be pollinated by hand within a cage or bee-proof sleeves that enclose part of a branch. This is impractical for breeding purposes and can only be used to generate a small number of fruits.

Cross-pollinated seedlings are obtained under similar conditions:

1. Two different cultivars in close proximity but at a distance from other avocado trees can be allowed to cross-pollinate. The closer the two cultivars are situated, the greater the likelihood of crossing. However, there are differences in the effectiveness of cross-pollination between different cultivars (Degani et al. 1990) and progeny have to be identified by molecular markers (see below).
2. Flowering branches or trees of the pollinator can be enclosed in a cage with bees. This method will produce a variable mixture of crosses and selfers (Degani and Gazit 1984; see 1 above).
3. Seedlings can be cross-pollinated by hand inside cages or sleeves. This technique should produce only crosses, but conceivably both selfing and out-crossing could occur. This method (successful only under specific conditions) will produce a very limited number of progeny, definitely insufficient for breeding purposes.

None of the above methods can ensure the identity of the progeny which would mostly be mixtures of selfing and crossing. Whenever important to know the seedling identity, molecular markers have to be applied. For isozymes, see Degani et al. (1990); Torres et al. (1978); Goldring et al. (1985). For DNA markers, see Lavi et al. (1991a). The only exception is truly isolated trees (or a commercial block of a single cultivar), which would give rise only to selfers.

Based on our experience, we do not recommend controlled pollinations or identification of progeny by molecular markers for breeding purposes since the breeding

objectives of avocado are "general" (see Section 8.4). We have to ensure the maximum genetic variation of the seedling population and this could be achieved by open-pollination. On the other hand, progeny identification is required for genetic studies.

8.7.2.2 Open-Pollination

Seeds are collected from the selected tree(s) and depending on the degree of isolation and the cultivar, various proportions of out-crossed progeny can be produced. The advantage with this approach is its low cost permitting the rapid evaluation of many seedlings. The disadvantage is that little is learned of the inheritance of commercial traits.

8.7.3 Increasing Seed Set

Hand-pollination is impractical for breeding programmes due to the high cost and the low fruit set. Schroeder (1958) cross-pollinated over 10,000 'Fuerte' flowers and only 4 mature fruits were obtained. Eisenstein and Gazit (1989) reported a yield of 1.3–27.2% fruitlets in hand-pollination of various crosses.

A number of techniques can increase fruit set from hand-pollination:

1. Select trees growing in an optimal location and provide a high level of management (Bergh 1967) (e.g. irrigation, nutrition, disease and insect control and wind protection).
2. Avoid excessive shading by using a screen material that transmits as much light as possible and remove limbs shading the breeding site (Lahav 1970).
3. Hybridise or self-pollinate in the productive ('on') year for that tree, since all avocado trees are alternate bearing to some degree.
4. Girdling maximises fruit yield and especially fruit number. Girdling after fruit set may increase its chances of fruitlets' survival (Lahav et al. 1971, 1972).
5. Select a heavy-setting cultivar as the seed (maternal) parent.
6. Use a potent pollen parent (Gafni 1984).
7. Cross-pollinate cultivars belonging to complementary flowering groups (Lahav and Gazit 1994) that flower at the same time.
8. Pollinate during optimal weather conditions.

8.7.4 Growing the Seedlings

An avocado seed left at ordinary room temperature and humidity remains viable for only a few days after its removal from the fruit as the embryo has no protection against desiccation (Storey et al. 1986). However, avocado seeds remain viable for up to 15 months when stored at 5 °C at high humidity (Halma and Frolich 1949). Humidity can be easily maintained in storage by placing seeds in sealed

Table 8.4 Seedling productivity as related to their grafted duplicates (±SE)

Number of Seedling Evaluated	Evaluation of Seedling Productivity	Evaluation of Graft Productivity
5	Low 1	2.3 ± 0.5
56	Low – Medium 2	2.6 ± 0.3
76	Medium 3	3.0 ± 0.3
59	Medium – High 4	3.5 ± 0.3
33	High 5	4.4 ± 0.6

After Lahav et al. (1995), with permission.
Productivity was evaluated from 1 (low) to 5 (high).
$R = 0.55^{***}$ (The three asterisks indicate level of significance $P = 0.001$)

polyethylene bags. Details on seed treatment, storage and germination procedures were summarized by Bender and Whiley (2002).

Under optimal growth conditions (night-day temperature range of about 23–25 °C), most viable seeds will germinate within a month (Alexander 1977). Vigorous seedlings will attain a height of about 1 m in three months, while less vigorous seedlings may require six months to reach a suitable size for field planting. Cooler temperatures greatly retard seedling growth.

Growing seedling progeny on their own roots is cheaper, but under some conditions (such as saline water or root rot) it may be necessary to graft onto salt resistant or Phytophthora tolerant rootstocks. About half the Israeli progeny from breeding programmes are grafted due to salinity conditions. Avocado seedlings and their grafted duplicates showed no significant performance differences (Lahav et al. 1995) and have a high and significant similarity in productivity (Table 8.4). Horticultural approaches aimed at economising field space have been discussed above under 'Length of the juvenile phase'. Detailed instructions with illustrations on avocado propagation were reviewed by Whitsell et al. (1989) and Bender and Whiley (2002).

The most efficient seedling spacing is the closest that permits adequate fruitfulness. This will vary with the average vigour of each seedling group. Suitable planting distances may be 1–2 m in the row and 4–6 m between rows (Fig. 8.4). Generally, the close planting distances increase the juvenile period and should therefore be avoided. At the closer spacing some of the smaller trees may have their fruiting delayed by shading, but this can be relieved through gradual reduction in tree density by removing inferior seedlings. The breeding block is replaced with new seedlings after 6–8 years, which is ample time for individual seedlings to exhibit the major breeding objective of precocity, but allowing a safety margin to cover delayed fruiting due to genetic or environmental factors. Recommended management techniques for nursery and field planted seedlings are summarized by Bergh et al. (1996).

8.7.5 Assessment of the Seedlings

A detailed list of avocado descriptors was prepared by the International Plant Genetic Resources Institute (1995). In the California breeding programme, recorded

Fig. 8.4 Avocado seedlings planted on ridges (two rows on each for drainage). Each seedling is protected against sunburn and frost by a mesh cylinder (from Bergh and Lahav (1996), with permission)

evaluations are usually made only on the seedlings selected for further testing, scoring only the important commercial traits. Trees are judged by size, shape, productivity and flowering. Fruit is evaluated by size, shape, colour, russet, attractiveness, time of maturity, skin thickness and roughness, seed size and tightness in the cavity, flesh attractiveness and fibre in the flesh (see Section 8.4.2.1). The ripened fruit is evaluated again in the laboratory focussing on flavour (nuttiness, sweetness, bitterness, other defects or comments), peeling ability and the time from harvest to softening (eating-ripe).

The Israeli breeding programme evaluated every seedling produced and records numerous parameters for genetic study, including nearly all of those listed for California plus the following:

1. *Tree*: The distance between buds, leaf and flush colour, flush lenticels and leaf size, shape, habit, margin waviness and anise scent.
2. *Flowering*: Time of year, intensity and flowering group.
3. *Fruit*: Length of inflorescence stalk (peduncle) and fruit stalk (pedicel), thickness of fruit stalk, its attachment position, suitability for snap picking (instead of having to cut the stem), skin gloss, seed surface, flesh texture, oxidation of cut fruit and shelf life.

Some traits are evaluated quantitatively by measurements but most are visually estimated. Evaluation of economic traits is summarized in Table 8.5 (Lahav et al. 1995). For genetic studies many traits can be assessed but for selection of new cultivars the assessment is usually limited to few economic traits.

It is currently recommended that two selection stages be performed. The first stage is aimed only at fruit assessment and is carried out in the first year of cropping. In the second stage, selected seedlings are grafted onto mature trees and are subsequently assessed for all fruit and tree characteristics under commercial conditions. This two-stage selection process ensures faster and efficient breeding outcomes (see Table 8.2).

Table 8.5 Evaluation of economic avocado traits

Traits	Evaluation Criteria
Tree size	Very big (1); Big (2); Medium (3); Small (4); Dwarf (5)
Flowering Intensity	Profuse (1); High (2); Medium (3); Light (4); Very light (5); None (6)
Flowering Time	Precocious (1); Early (2); Early mid-season (3); Late mid-season (4); Late (5); Very late (6)
Fruit Weight	In grams
Fruit Size Uniformity	High (1); Medium (2); None (3)
Fruit Shape Uniformity	High (1); Medium (2); Slight (3)
Fruit Density on the Tree	Dense (1); Medium (2); Light (3); Very light (4)
Damage by Snap-Picking	Minimal (1); Slight (2); Medium-severe (3)
Skin Thickness	Mexican type (1); Like 'Fuerte' (2); Like 'Tova' (3); Like 'Hass' (4); Like 'Nabal' (5); West Indian type (6)
Ease of Peeling	Excellent (1); Good (2); Medium (3); Difficult (4); Impossible (5)
Separation of Seed from Flesh	Easily (1); With some difficulty (2); Impossible (3)
Seed Weight (% of Fruit Weight)	< 6% (1); 6–10% (2); 11–15% (3); 16–20% (4); 21–25% (5); 26–30% (6);> 30% (7)
Taste Evaluation	Excellent (1); Very good (2); Good (3); Poor (4); Bad (5)
Darkening of Cut Surface (6 h After Cutting)	None (1); Slight (2); Severe (3)
Harvest to Softening Time (Room Temperature at About 20°C)	<6 days (1); 6–10 days (2); 11–15 days (3); 16–20 days (4); 21–25 days (5); 26–30 days (6);>30 days (7)
Shelf Life	In days

From Lahav et al. (1995), with permission.

8.8 The Genetic Basis of Fruit Abscission

Fruit abscission is a major problem that significantly affects yield. Moreover, it is the major reason for the low success rate of hand-pollination. Over the years, many explanations have been suggested for the occurrence of fruitlet drop by plant physiologists. In most cases, no horticultural practice has been successful in significantly reducing fruit drop, although shoot tipping in spring (Biran 1979) and the mid-anthesis foliar application of growth retardants (Wolstenholme et al. 1990) partly reduced it. Later it has been shown in Israel (unpublished data) that growth retardants reduced significantly fruit drop. Genetic selection was found to be an important factor in avocado fruitlet abscission. Thus, it was shown that the abscised fruitlets had different genotypes compared with the fruitlets that remain on the tree (Degani et al. 1986). This result suggests that abscission does not occur at random but depends on the seed genotype – selective drop.

Seedlings originated from self-pollinated 'Ettinger' trees that were caged under a net in the presence of a beehive were genotyped with leucine aminopeptidase (LAP), malate dehydrogenase (MDH), acid phosphatase (AP), glutamate oxaloacetate transaminase (GOT), phosphoglucomutase (PGM) and triosephosphate isomerase (TPI). Selfing was proved by MDH, AP and GOT. Segregation was analysed by PGM and TPI and resulted in the expected Mendelian ratio. On the other hand,

analysis of this population in the LAP-2 locus resulted in a significant deviation from the expected ratio. In another experiment, three 'Ettinger' trees were individually caged and fruitlets were sampled both from trees and after dropping at various stages of fruit maturity. Deviation from the expected Mendelian ratio was increased significantly during the late stages of fruit maturity. This experiment was repeated with similar results in the consecutive year. Among 48 mature fruits that were picked from an 'Ettinger' tree, 45 were FS, 3 were FF and none were SS (Degani et al. 1986).

Genetic selection, expressed in the high frequency of FS genotypes, the low frequency of the FF genotypes and the absence of the SS genotypes, is the most probable explanation for fruitlet abscission (Degani et al. 1986). This conclusion is supported by the fact that the SS genotype was found at the early stage of fruit development and never among mature fruits. It is believed that the LAP-2 locus serves as a genetic marker linked to some other locus having selective adaptation. One can assume that similar genetic selection might operate on other loci, thus explaining the massive fruitlet abscission in avocado. If so, this phenomenon has a major evolutionary impact allowing a 10^4-fold selection rate, i.e. 100–200 mature fruits from a potential of about 1,000,000 flowers per tree at each generation (Lahav and Zamet 1999), thus providing the avocado a large evolutionary flexibility by 'choosing' the most adaptable seeds.

8.9 Classical Breeding Systems

8.9.1 Selection of Naturally Occurring Superior Variants

Before the onset of avocado breeding programmes, every avocado cultivar being grown on a large scale throughout the world originated as a random seedling. 'Fuerte', which for decades was the leading cultivar in the Mediterranean/subtropical regions worldwide, is a good example. Introduced into California in 1911 as budwood from a seedling tree in Atlixco (Mexico), it gradually gained prominence. Later it was exported from California to Israel, South Africa, Chile, Australia, Mexico and other subtropical countries where it became a leading cultivar.

Many early California cultivars originated in Mexico or Central America, either from asexual propagation of selections made there or from imported seeds (Bergh 1957). The Guatemalan cultivars 'Benik', Itzamna' and 'Nabal' were introduced as budwood while 'Dickinson' came from imported seed. Later, introductions to California have been almost entirely limited to those collected in a search for resistance to Phytophthora root rot (Coffey 1987). These introductions have also been mainly from Central America and neighbouring countries where *Persea* species abound.

Seeds imported from California, Mexico and Central America formed the basis for the Florida industry (Ruehle 1963), which later became a source of germplasm

for tropical areas in the same way as California for the subtropics. Florida cultivars, some entirely of the West Indian race, others Guatemalan/West Indian hybrids, have been successful in various countries of Central America, South America and the West Indies, in coastal Mexico and in the tropical regions on other continents.

Several hundred random seedlings selected in California have been named and five ('Hass', 'Pinkerton', 'Reed', 'Bacon' and 'Zutano') are currently grown on a commercial scale. Similar local randomly selected seedlings have achieved some commercial success in other countries, notably 'Ettinger', and to a lesser degree 'Horshim' in Israel and 'Sharwil' in Australia and Hawaii.

In nearly every region where avocado is grown, local seedlings have been selected and named. Note that numerous mediocre-quality cultivars aggravate marketing problems. Since modern avocado industry is based on grafted trees and no superior somatic mutation had been reordered in avocado, we consider the chance to detect naturally occurring superior cultivars to be quite small.

8.9.2 Inter-specific Crosses

Attempts have been made to generate resistant rootstocks by inter-specific hybrids of avocado with *Persea* species immune to the disease. Up to the present time, all such crosses have been unsuccessful. Both graft and cross incompatibility appear to be complete between the sub-genus *Persea*, which includes the avocado, and sub-genus *Eriodaphne*, which includes all the known immune species.

Within the sub-genus *Persea*, 'Martin Grande' (G755) appears to be a natural hybrid between *P. americana* var. *guatemalensis* and *P. schiedeana* (Ellstrand et al. 1986; Furnier et al. 1990). 'Martin Grande' has about as much resistance to *Phytophthora cinnamomi* as any compatible line known. However, production from trees grafted to this rootstock has been poor in several countries where it has been evaluated (Whiley et al. 1990).

P. floccosa Mez has the valuable trait of setting much larger numbers of fruits than do other taxa in the *Persea* sub-genus. However, its fruits are very small and the seed relatively large. It has been hybridised with several large-fruited, small-seeded cultivars and a few of the better F_1's have been selfed or "backed crossed" to commercial cultivars. The results have not been promising. Heavy setting ability has been lost at least as rapidly as commercial quality has been approached and flavour has been mediocre at best.

Inter-species hybridisation by somatic hybridisation and genetic engineering is discussed by Pliego-Alfaro et al. (2002).

8.9.3 Mutations and Polyploidy

Occasional spontaneous mutations have long been recognised in the avocado. Tree shape, leaf size, shape and colour, fruit size and shape or skin surface and thickness

have been clearly different on certain 'sported' limbs. While trees of a number of cultivars have been affected, 'Fuerte' appears to be the most unstable with a pronounced tendency for somatic mutation. Several such mutations as 'Weisel', 'Newman' and 'de Bard' (Hodgson 1945) have been selected for commercial production. In contrast, 'Hass' seems to be comparatively stable. No mutation has yet proven horticulturally beneficial.

Somatic mutations affecting a quantitative trait like yield are much more difficult to detect, especially because of the highly erratic nature of cropping. Good evidence for genetically determined yield differences was first obtained for 'Fuerte' (Hodgson 1945). In addition, large-scale studies in Israel raise the possibility that there are 'Hass' variants differing in their yield (Ben-Ya'acov 1973). The sound nursery practice of taking buds from limbs of demonstrated high-yielding ability guards against detrimental mutations.

In the hope of enhancing its moderate root rot resistance, 'Duke' scions were irradiated with fast neutrons in the California programme. One resulting selection 'D9' was tested as a commercial stock because of its considerable root rot resistance and somewhat dwarfing impact. 'D9' was found more productive than 'Martin Grande' but less than 'Borchard' and 'Duke 7' (Arpaia et al. 1992). A radio-induced mutation-breeding programme with ^{60}Co gamma rays was conducted in Mexico by De la Cruz et al. (1999). They reported higher number of fruits/tree in some of the irradiated trees. Bringhurst (1956) generated tetraploidy by colchicine in 'Fuerte' and 'Mexicola'. He reported some gigas characteristics in the vegetative organs but fruit set was reduced to almost nil.

8.10 Genetic Analysis

8.10.1 Qualitative Traits

The genetics of fruit skin colour, flowering group and anise scent was studied in breeding populations of 1,699 seedlings (Lavi et al. 1993a). The three traits were recorded over a two year period. Parent cultivars included: 'Anaheim', 'Ettinger', 'Fuerte', 'Hass', 'Horshim', 'Irving', 'Nabal', 'Pinkerton', 'Reed', 'Regina', 'Rincon', 'Rosh-Hanikra II', 'Tova' and 'Wurtz'. Isozyme analysis was used to distinguish between hybrids and self-pollinated seedlings (Degani and Gazit 1984). However, the possibility that some seedlings were wrongly classified cannot be ruled out.

In all types of crosses the average X/Y ratio (X and Y being green or purple skin colour, A or B flowering group and (+) presence or (–) absence of anise among the progeny was one or higher with a wide variation in the ratio between crosses (Table 8.6). It is interesting that selfing in each trait (fruit skin colour: green × green or purple × purple; flowering group: B × B or A × A; and anise scent: (−) × (−) or (+) × (+) resulted in more progeny of the first phenotype (green skin, flowering group B and no anise scent) (Lavi et al. 1993a). These results rule out the

Table 8.6 Progeny distribution in three avocado traits

Traits	Crosses	Progeny Phenotype		Ratio
Fruit Skin Colour		Green	Purple	Green/Purple
	Green × green (selfings)	121	14	8.6
	Green × green (crosses)	273	20	13.6
	Green × green (total)	394	34	11.6*
	Green × purple	10	4	2.5
	Purple × green	71	48	1.5
	Purple × purple (selfings)	5	4	1.2
	Total and weighted mean	480	90	5.3*
Flowering Group		B	A	B/A
	A × A (selfings)	11	9	1.2
	A × B	148	111	1.3
	B × A	32	17	1.9
	B × B (selfings)	35	13	2.7
	Total and weighted mean	226	150	1.5*
Leaf Anise Scent		No anise	Anise	No anise/Anise
	Anise × anise (selfings)	225	29	7.8
	Anise × anise (crosses)	57	24	2.4
	Anise × anise (total)	282	53	5.3*
	Anise × no anise	59	11	5.4
	No anise × anise	555	119	4.7
	No anise × no anise (selfings)	257	17	15.1
	No anise × no anise (crosses)	270	65	4.2
	No anise × no anise (Total)	527	82	6.3*
	Total and weighted mean	1423	265	5.4*

From Lavi et al. (1993a), with permission.

*Weighted mean

possibility of a single gene coding for these traits. The results could be explained by the assumption that the traits are coded by several loci with several alleles in each while the various phenotypes may result from various heterozygous combinations. Furthermore, it was suggested that the inheritance of these traits is based on a threshold value beyond which the phenotype shifts from one phase to another as suggested by Carter (1969).

8.10.2 Quantitative Traits

Hybridisation is the only way to combine complementary desirable features of different cultivars and the most efficient way to obtain a desirable intermediate trait when the available breeding materials have extreme phenotypes. For example, the commercially important ‘Booth’ numbered selections in Florida are evidently natural hybrids of the Guatemalan and West Indian races and are intermediate in the harvesting season as well as other useful traits. Parallel inter-racial hybridisations between Guatemalan and West Indian types have given rise to important

intermediate cultivars in Hawaii. The assumption behind hybridisation is that most of the genetic variance is additive and therefore combining alleles from two parents will result in offspring having the desired performance (Hansche 1983). An analysis of several quantitative traits in avocado was conducted in order to estimate the variance of components and their heritability. It was shown (Lavi et al. 1993b) that genetic variance (both additive and non-additive) is large for most avocado traits. In four of the nine traits (tree size, flowering intensity, fruit density and inflorescence length), relatively large non-additive variances in components were detected. In the second more detailed stage of this study, 14 cultivars were used to carry out 12 crosses and 12 selfings. The number of progeny varied between 2 and 431 seedlings per each self or cross and the total population consisted of 1,938 seedlings. The parent cultivars represented much of the variation that exists within avocado. Parentage verification was based on isozyme analyses. Only seedlings of known parentage were used in this study and the traits were assessed by measurements or by visual scoring with results averaged for 2–5 years.

The value of the non-additive genetic variance was significantly higher than zero for all traits (anise scent, fruit density, flowering intensity, fruit weight, harvest duration, inflorescence length, seed size, softening time and tree size). These values ranged from 36.3% in time to softening to 49.3% in harvest duration. In contrast, the additive genetic variance was non-significant in all traits. However, significant environmental variance was present and except for flowering intensity the estimate accounts for 33–35% of the total phenotypic variance. The values of the narrow sense heritability (h^2n) ranged from 0 in fruit density and flowering intensity to 0.5 in seed size and 0.48 in softening time (Lavi et al. 1993b).

These estimates indicate that non-additive genetic variance is a major component of the total genetic variance and is significantly greater than zero in all traits. The high level of heterozygosity known to exist in avocado (Lavi et al. 1991b) could explain the prevalence of a large non-additive (dominant) genetic variance (Fisher 1930). Thus, these results explain the common frustration after crossing two cultivars and obtaining a wide range of phenotypes among the progeny (rather than the naive expectation of combining traits from both parents). It must be emphasized that these conclusions are limited to the set of cultivars used in this study. The importance of the broad sense heritability (h^2b) originates from the fact that best performing seedlings in the selection plots could result from either environmental or genetic factors. The higher the value of the h^2b, the greater the confidence that genetic factors are responsible for performance. In such cases, a few grafted trees must be generated from each selected seedling for the next phase of evaluation. The choice of parents for breeding depends, first of all, on the breeding objective. In the case of the avocado, the main objective is to obtain new cultivars better than those currently available (see Section 8.4). Since the major variance components in most traits important to breeders are non-additive, parents should be chosen in order to maximise the genetic variance in the progeny. Thus, the chance to obtain the desired combination of genes and alleles is increased. This should be achieved by choosing a broad spectrum of parent cultivars and may include some with inferior performance.

8.11 Genetic Markers

8.11.1 Isozymes

Isozymes have been used mainly to assess the level of cross-pollination. For additional information on this topic, see Gazit and Degani (2002).

8.11.2 DNA Markers

Development of DNA markers (Botstein et al. 1980) paved the way towards new applications of this tool mainly due to the high level of polymorphism and the abundance of these markers. Several classes of DNA markers were developed to show existing polymorphism between individuals of the same species as well as between species. Several classes of these DNA markers were applied to avocado including RFLPs, Rapid Amplified Polymorphic DNA (RAPD) and Variable Number Tandem Repeats (VNTR) (DFP and SSRs). These markers have been applied to obtain various goals (Furnier et al. 1990; Pliego-Alfaro et al. 2002; Scora et al. 2002).

8.11.3 Level of Heterozygosity

Two types of VNTR markers were used for estimating the level of heterozygosity (Lavi et al. 1994b). Multilocus DNA markers were used to analyse avocado progeny resulting from either crossing or selfing cultivars. In five crosses, the heterozygosity level was found to be 100%, while in two self-pollinated families, heterozygosity was 90% and 94%. Typing of 59 loci with SSR markers in 5 avocado cultivars revealed an average heterozygosity (AH) of 0.58 ranging between 0.50–0.66 (based on Nei and Roychoudhury 1974); $AH = 1 - \sum pi^2$, where pi is the frequency of the ith allele. Gene Diversity (GD) varied between 0.42 and 0.66 calculated according to Rongwen et al. (1995); $GD = 1 - \sum pi^2$, where pi is the frequency of the ith pattern. Percentage heterozygosity calculated as the proportion of heterozygous genotypes from all those tested, varied between 38 and 70%. The percentage of fragments that exhibited Mendelian inheritance was 62.5–85% ($P < 0.05$) for DFP fragments and 85% for the SSR alleles.

Both RFLP and isozyme markers show low levels of heterozygosity compared to the above-mentioned results. This is due to the nature of the VNTR markers (Lavi et al. 1994a). A low level of self-pollination is probably the cause for the high level of heterozygosity in avocado (Lavi et al. 1993b; Mhameed et al. 1996).

The (AG)n markers were found to be the most efficient among the SSR markers analysed in avocado having heterozygosity levels of 0.58–0.70. Analysis of 11 cultivars with 17 SSR markers revealed on average 6.1 alleles per marker, an AH level of 0.79 and an average GD level of 0.78.

8.12 Genetic Linkage Map

SSR markers were generated by screening a genomic library of avocado having short DNA inserts (about 500 bp). The library was screened with all the dinucleotides and some of the tri- and the tetra-nucleotides. (A)n and (AG)n were the most frequent to occur. The total number of micro-satellites in the avocado genome was estimated to be about 45,000. From this library, 238 positive colonies were isolated and their sequence was determined. In 113 colonies a sequence defined as SSR was found and primers to the flanking regions were synthesized in 62 of them (Sharon et al. 1997). These 62 markers, together with 30 other markers synthesized earlier (Lavi et al. 1994a) and an additional SSR marker synthesized on the basis of a GeneBank sequence to the avocado cellulase gene were applied to several analyses.

Fifty offspring of the cross 'Ettinger' × 'Pinkerton' were genotyped with 93 SSR markers (Sharon et al. 1997) of which 51 were found to be polymorphic and reliable. Ten markers were inherited in a non-Mendelian fashion (4 with a significance level of $P \leq 0.01$). These markers together with 17 polymorphic RAPD markers (identified by screening 100 RAPD markers) and 23 DFP markers were used to draw a genetic linkage map of avocado. Two-point analysis resulted in 29 linked marker loci (LOD score ≥ 3). The map consists of 12 linkage groups having 2–5 markers per group (a total of 35 markers) covering 357.2 cM. Comparison of the parental with the maternal map shows that the crossing-over frequency was on the average 21% higher in the maternal parent ('Pinkerton'). No linkage was detected between SSR and DFP markers and within RAPD markers. Based on the Poisson distribution, the markers were found to be randomly allocated to the linkage groups.

Note in proof. Michael Clegg of the University of California, Riverside, has recently started a project aiming at generation of additional SSR markers and identification of linkage between these markers and genes controlling traits of interest to the avocado industry (Clegg 2005). This project contains several aspects including phenotyping of important traits in experimental trees, estimation of heritabilities and genotyping of out-crossed avocado progeny by seven SSR markers. More results are expected in the next two years of this project.

8.13 Modern Breeding Methodologies

8.13.1 Cryopreservation

Efendi and Litz (2003) have developed two cryopreservation procedures for avocado: (1) Slow cooling at −1 °C/min from 25 °C to −80 °C followed by rapid cooling to −196 °C; (2) Rapid cooling from 25 °C to −196 °C. All tested embryonic cultures that recovered from the cryogenic storage grew normally. Cryopreservation is an alternative method for long-term conservation of avocado genetic resources which have so far been maintained ex situ in field repositories at high cost and under continuous various environmental threats. Cryopreservation is expected to improve and efficiate avocado breeding programmes.

8.13.2 Generation of Rootstocks by Somatic Hybridisation

Resistance to *Phytophtora cinnamomi* was identified in several *Persea* spp. within the sub-genus *Eriodaphne* (Bergh et al. 1996). These species include *P. borbonia*, *P. cinerascens* and *P. pachypoda*, which are all sexually and graft incompatible with avocado (*P. americana*). Thus Litz (2005) suggested overcoming this incompatibility by the fusion of avocado (*P. americana*), protoplasts with protoplasts of resistant *Persea* spp. The same procedure could obviously be applied to generation of rootstocks harbouring other horticultural important traits.

Putative somatic hybrids have been recovered by this approach although at a very low frequency of less than 0.001% (Witjaksono 1997; Witjaksono and Litz 1998, 1999). A newer approach for somatic fusion was developed by Litz (2005). This procedure is based on callus cultures that have been initiated from stem segments of micro-propagated plantlets generated from various resistant *Persea* species.

Somatic embryos from inter-specific hybridisations of *P. americana* (avocado) and *P. cinerascens* were recovered several years ago based on morphological markers. Some of these hybrids began to germinate in vitro. Further data regarding these hybridisations are not available at this time.

8.13.3 Propagation of Avocado via Tissue Culture

Success with in vitro propagation of avocado has been achieved through saving aborted embryos (Sedgley and Alexander 1983). The embryo culture medium stimulated the production of shoots, which were then micro-grafted to rootstocks. This method may be useful for rescuing especially valuable hybrids or selfs, but no report of its application is available.

8.13.4 Tissue Culture and Transgenic Plants

Due to the difficulties in classical avocado breeding, a generation of transgenic avocado trees having the desired traits is obviously a major goal that will make avocado breeding of both cultivars and rootstocks much more efficient. In order to achieve this goal there is a need to overcome two main obstacles:

1. Development of a regeneration protocol that will allow gene transfer to avocado.
2. Availability of the genes, which control the important traits.

For a list of avocado genes, which have been isolated and the gene transfer methodology for avocado, see Pliego-Alfaro et al. (2002). The following is a short summary of this subject:

A transformation system for the generation of new cultivars has to be based on the ability to transform existing cultivars. In other words, there is a need for a regeneration system from explants taken from mature trees. Available regeneration systems, which are based on either juvenile material or embryo cultures, do not serve this

purpose. Only regeneration from mature material would serve breeding purposes, either for large-scale propagation of rootstocks with resistance to Phytophthora root rot and other desired traits or for the production of new cultivars (Pliego-Alfaro and Bergh 1992).

An embryonic avocado culture (derived from immature zygotic embryos of 'Thomas') was transformed using *Agrobacterium tumefaciens*. The reporter genes were GUS and nptII. The maturation of the transgenic embryos was achieved but regeneration to mature plants was not successful (Cruz-Hernandez et al. 1998).

Rahajo et al. (2003) reported the transformation of avocado with various genes (see below). Embryonic cultures were induced on semi-solid medium and transferred into liquid medium. The embryonic suspension cultures were transformed with *Agrobacterium tumefaciens* using the vector pGPTV containing glyphosate resistance and the CaMV 35S promoter. The transformation experiments included: 'Gwen' with the genes Chalcone synthase and nptII (as a selection marker) and 'Hass' with the antifungal protein (AFP) and the glyphosate resistance. Transformed plants with the genes: AFP; AFP+Chalcone synthase and SAMases (a bacterial gene mediating the breakdown of S-adenosylmethionine – SAM (which is precursor of ethylene and thus can delay ripening) and ACC deaminase (degrading ACC – a precursor of ethylene) have been regenerated by micro-grafting. Plants transformed with the AFP are reported to be assessed in greenhouse. No further information is available at the time of writing this chapter.

Although we consider the production of transgenic plants as a significant breakthrough in avocado breeding, we are aware of the major difficulties currently preventing the achievement of this goal. At present, the use of classical breeding techniques is the only available way to generate new cultivars. We believe that there is a simultaneous need to improve the efficiency of classical breeding and to develop modern breeding technologies.

8.14 Linkage Between DNA Markers and Loci Controlling Important Traits

Genetic linkage between DNA markers and genes controlling important traits could be applied to improve breeding projects. This is achieved through Marker Assisted Selection by selecting for the marker rather than for the trait and thus making breeding more efficient by saving time and space. In the long term, this linkage can be used for isolation of the genes responsible for these traits (Tanksley et al. 1995).

Only a few reports of such linkage are available for avocado that is lagging behind as compared with other species. Association between DFP fragments and 16 avocado trait loci was tested in two families by one-way analysis of variance and multiple regression (Sharon et al. 1998). The DFP fragments P4, P8, E2 and E5 in 'Pinkerton' × 'Ettinger' progeny were found to be associated with harvest duration, skin colour, skin thickness and skin surface respectively. The fragments P1, P8, B1 and B4 in the 'Pinkerton' × 'Bacon' progeny (half sibs of the first population) were

found to be associated with fruit weight, skin colour, seed size and peeling respectively. Based on the two populations, the fragment P8 was found to be associated with the black-purple fruit skin colour. The intensity of this fragment in the DNA pools of progeny having green skin colour compared to those having black-purple skin colour supported this association. These results are interpreted as an association and maybe genetic linkage between the DNA fingerprint fragment P8 and locus (i) regulating avocado fruit skin colour.

The multi-locus markers are very useful for identification purposes but less so for linkage analysis. For this purpose the single locus VNTR markers are better suited. These markers are based on micro-satellite sequences flanked with conserved sequences. Primers based on these flanking sequences allow the use of Polymerase Chain Reaction (PCR) for their genotyping. These SSR markers are very polymorphic, very abundant and very reliable and thus served as the marker of choice for the human genome. SSR markers have been successfully used in several plants (Akkaya et al. 1992) including the avocado.

Sixty progeny of the 'Pinkerton' × 'Ettinger' cross were analysed to identify linkages with loci coding for agriculturally important traits (Sharon et al. 1998). One-way analysis of variance resulted in the identification of linkages with seven of the nine analysed traits. High levels of significance ($P \leq 0.01$) were detected in the traits. For example, skin gloss was linked to two SSR markers ($P = 0.0014$); seed size was linked to one SSR marker ($P = 0.0006$); and the amount of fibre in the flesh linked to SSR markers on linkage group 3 (especially with the marker AVAO4) ($P = 0.00001$). The application of Interval Mapping to allocate loci coding for these traits resulted in three cases where the LOD score value was ≥ 2 (skin gloss in linkage group 6; skin surface in linkage group 9; and fibres in linkage group 3). Further analysis revealed allelic interaction in the locus (i) controlling fibres in the flesh. The level of significance was found to be very high in the marker locus AVAO4 in certain genotypes (Sharon et al. 1998). These results point towards the potential of using SSR markers in genetic studies and the benefits of using this technology in breeding fruit trees such as avocado.

We are not aware of application of SNPs – which lately became the marker of choice in human and other species – to avocado.

8.15 Achievements

Many new avocado cultivars are currently available. However, we will limit this discussion to those cultivars from breeding programmes that have attained (or show clear promise of attaining) commercial significance. At the moment, the Californian and Israeli avocado breeding projects are in their final stage of evaluation of new and better performing progenies from already grown seedlings. The major objectives in both programmes are to find better than 'Hass', black skin cultivars and cultivars from the B flowering group that might increase productivity of 'Hass'. Selections of outstanding rootstocks and cultivars exist in South Africa and Australia, and a small scale breeding project in Mexico.

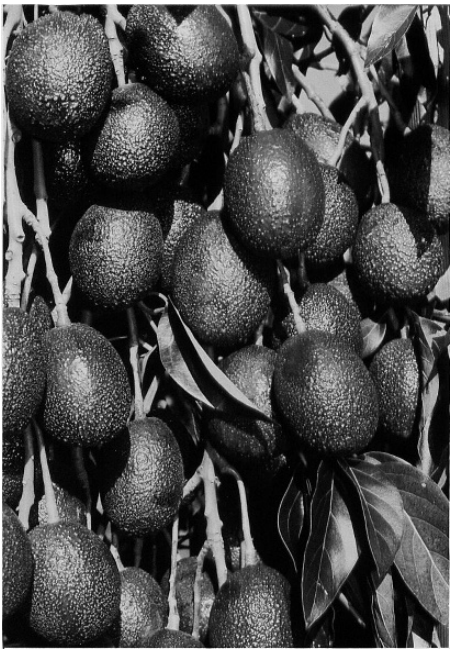

Fig. 8.5 Heavy set on 'Gwen' trees, 19 months after top-working

'Gwen' is a selection from the University of California avocado breeding programme (Bergh and Martin 1988; Martin and Bergh 1988, 1989). It is a seedling of 'Thille', which is in turn a seedling of 'Hass'. Propagated as either nursery trees or top-worked in the field, it may out-crop 'Hass' several fold in the early years of fruiting (Fig. 8.5) and about twofold indefinitely. However, it has not shown this level of production everywhere. Its smaller tree size makes picking cheaper. The length of its season is similar to 'Hass', but starts about a month later. In California, its flavour is superior to 'Hass' over most of its season.

'Lamb Hass' (BL 122) is another product of the California avocado breeding programme. It is a 'Hass'-like cultivar that produces 50% more fruits as compared to 'Hass' trees of similar age and growing conditions (Martin 1993). 'Lamb Hass' is a precocious, consistent bearer, which holds fruits very late in the season even in fierce wind conditions.

'Sir Prize' (4-18-15) is a selection from the University of California avocado breeding programme that is an early season cultivar with a green skin. Skin thickness is similar to that of 'Fuerte' although somewhat pebbly. Peeling is good and fruit quality excellent (Martin 1993). 'Sir Prize' is a good producer but has an extreme alternate bearing habit. It belongs to the B flowering group cultivars and shows promising results as a pollinator to 'Hass' (Arpaia 2004).

Some of the newer promising University of California releases are 'Harvest' (Fig. 8.6), a very heavy 'Hass'-like producer with excellent shelf life and 'Gem'

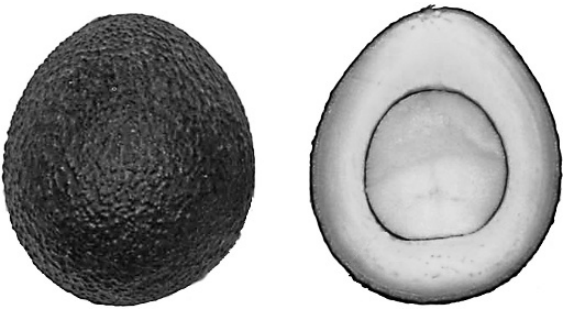

Fig. 8.6 "Harvest", a 'Hass'-like heavy producer, new release from the California breeding programme (from M.L. Arpaia, with permission) (*See* Color Insert)

(3-29-5) (Fig. 8.7) a good producer that shows less tendency to alternate bearing than most of the other selections (Witney and Martin 1998; Arpaia 2004).

'Ardith' (OO-28) was selected by the California breeding programme, but has only been commercialised in Israel. The tree is medium-sized and spreading. It is an oval, green skinned fruit of 200–300 g with a small seed. The fruit is late maturing, achieving excellent flavour only at the end of the 'Hass' season (Blumenfeld and Elimeleh 1986).

Two other Californian selections that have only been commercialised in Israel are the green, highly productive, mid-season 'Fino' (TX 531) and the 'Hass'-like but much larger in fruit size 'Ace' (T 142).

'Iriet' is the first cultivar generated by the Israeli avocado breeding programme (Lahav et al. 1989). It is a progeny of 'Hass' and an unknown pollen donor producing a small-medium tree. The fruit is pyriform, 300–500 g and glossy black (Fig. 8.8), with a very small seed and an excellent nut-like flavour. The season of maturity is late.

Fig. 8.7 'Gem', a new cultivar with reduced alternate bearing habit, released from the Califronia breeding programme (from M.L. Arpaia, with permission) (*See* Color Insert)

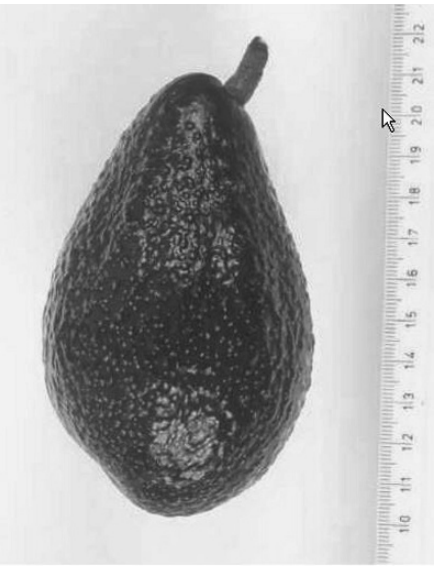

Fig. 8.8 'Iriet', a cultivar excellent in taste, from the Israeli breeding programme (*See* Color Insert)

'Eden' (Lavi et al. 1997) resulted from a cross in Israel between 'Pinkerton' and 'N-151-2' (a UC progeny). This was verified by analysis of mini-satellites and SSRs. It is a precocious producer with uniform dark green ovate fruit of 250–400 g.

'Galil' selected in Israel, is a green-skinned, Mexican type with a long neck (Fig. 8.9) and is an open-pollination seedling of 'Oshri' (a local selection). It is the earliest maturing summer cultivar harvested before 'Ettinger' in a season of great demand for avocado (Lahav et al. 1998).

'Arad' is an Israeli seedling of 'XX 102' that was caged with 'N-151-2' (both UC progenies). The 300 g fruit is green and harvested in mid-season. 'Arad' excels in precocity carrying many fruits already in the second year after grafting (Lahav et al. 2005).

'Lavi' is a seedling originated in Israel from a 'Hass' tree pollinated by an unknown donor (Regev et al. 2005). The tree is smaller than 'Lamb Hass' and its productivity is good. The fruit is more ovate and the peel thicker than 'Hass'. 'Lavi' was found to be especially interesting since its fruit is larger than 'Hass'.

'Naor' is an Israeli seedling of self-pollination of 'Horshim'. It is one of the most interesting selections since the fruit is very similar to 'Hass' but significantly larger in size (260–360 g). The harvest season is somewhat shorter than 'Hass' (Regev et al. 2008a).

Some interesting seedlings are currently under evaluation in Israel. One of them is 'Moti' (137–18), a seedling of 'R27T27' (originated from Hawaii) pollinated by unknown donor. The fruit is green, relatively large sized (350–400 g) and harvested after mid-season (Regev et al. 2008b). It excels in productivity but has tendency to alternate bearing. Another interesting seedling is 'Bar' (30–10) which is

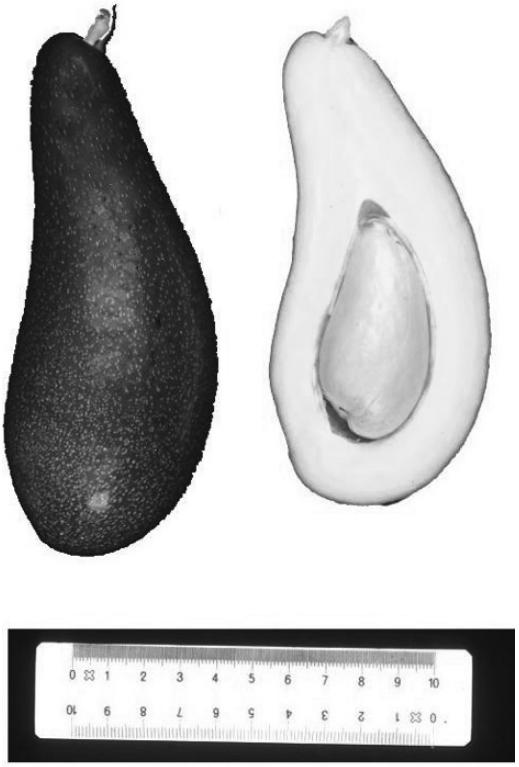

Fig. 8.9 'Galil', a very early maturing cultivar from the Israeli breeding programme (*See* Color Insert)

a 'Hass'-like cultivar but belongs to the B flowering group and has the potential to pollinate 'Hass' (Regev et al. 2008c).

8.16 Conclusions

Prospects for a significant increase in avocado consumption are bright since the fruit is exceptionally nutritious and as yet is quite unknown to many potential consumers worldwide. The future of avocado breeding is assured since past breeding programmes have so far barely scratched the surface of accessible genetic variation. This is even truer in most tropical regions where inferior local seedlings predominate, thus providing immense scope for improvement.

Based on our own experience, we offer the following recommendations for breeding avocado:

1. Unless a specific objective is required, open-pollination is preferred to controlled-pollination because the latter is more expensive and has no advantage.

2. In order to achieve an efficient breeding process we propose two selection stages. The first stage is carried out as soon as possible (even after one year of fruiting) on seedling populations (from either controlled crosses or open-pollination). This stage is aimed at identifying the seedlings' performance regarding fruit traits only. At this stage about 1% of seedlings are selected. The first stage lasts for about 6 years for progeny from open-pollination or about 11 years for controlled crosses. In the second stage, selected seedlings from stage one are grafted onto two or more mature trees in several locations (depending on the breeding goals) and managed under commercial orchard practices. At this second stage, the selected seedlings are assessed for yield, shelf-life and suitability for various climatic and soil conditions. At this second stage, selected seedlings are assessed by a wide team of growers, extension officers and breeders. Grafted trees of the most promising selections are prepared for the next stage of semi-commercial plots. The second stage is 4–5 years long.
3. High density planting should be avoided especially in the breeding orchard in order to shorten the long juvenile period.

The rapid advances in molecular biology, somatic hybridisation and other aspects of biotechnology have opened up new approaches in avocado breeding. The recent development of genetic markers for avocado and their applications to the classical breeding offers tremendous potential for avocado improvement. The introduction of specific genes for disease resistance from wild species into popular cultivars should be a reality in the foreseeable future. All these new technologies open new horizons to avocado breeding.

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Chapter 9
Cashew (*Anacardium occidentale* L.) Breeding: A Global Perspective

J.R. de Paiva, L. de M. Barros, and J.J.V. Cavalcanti

9.1 Introduction

The cashew tree (*Anacardium occidentale* L.), which first originated from Brazil, is now found throughout the tropical world and in some subtropical areas with favourable climatic conditions: rainfall not so heavy and a dry period between rainy seasons. Its economic importance lies in the commercial value of the kernel extracted from the fruit, one of the tastiest and much appreciated by the nut consumers. The cashew nut producing chain provides employment and income for thousands of people and a significant turnover for agri-business dependent countries (Barros and Crisóstomo 1995).

The Cashew was introduced from Brazil into India by the Portuguese during the sixteenth century. Following its introduction into southwestern India, the cashew probably diffused throughout the Indian subcontinent. Cochin served as a dispersal point for Southeast Asia as well (Johnson 1973).

A look at the evolution of the world production of cashew nut gives an understanding on how the geography of the production is changing fast. From the early 1990s, a number of Asian countries have become important producers that did not appear in the statistics previously (Table 9.1). This is a clear indication that unless the traditional producers change the systems of production in utilisation through adding technologies capable of increasing production and productivity, very little will actually be achieved to improve its capability to compete with Asiatic producers in the international market.

The most important nut producers are Vietnam, India, Brazil, Nigeria and Tanzania. Collectively, these countries account for 78% of the world production, according to FAO' statistical data from 2004. Vietnam has seen dramatic increases in production over the last 10 years, rising from 202,000 mt in 1995 to approximately 675,000 mt in 2004. This represents 31% of the world production

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Table 9.1 Cashew nut harvested area(ha) evolution in the main producing countries

	Year[1]									
Countries	1970	1980	1990	1995	1999	2000	2001	2002	2003	2004
Brazil	n.a.	n.a.	582,818	699,936	612,735	651,169	638,556	665,014	673,776	680,551
India	281,171	447,376	530,869	577,000	706,000	686,000	720,000	730,000	730,000	730,000
Indonesia	n.a.	116,808	125,000	190,000	250,000	260,000	260,000	260,000	260,000	260,000
Mozambique	290,000	130,000	40,000	55,000	66,000	50,000	50,000	50,000	50,000	50,000
Nigeria	40,000	40,000	50,000	155,000	278,000	291,000	291,000	321,000	321,000	321,000
Tanzania,	186,000	70,000	35,000	60,000	85,433	90,000	90,000	90,000	90,000	90,000
Viet Nam	3,000	8,000	140,000	189,400	143,700	151,000	204,600	240,200	257,900	260,000
World	855,132	905,473	1,721,090	2,296,768	2,792,312	2,855,116	2,915,011	3,024,938	3,053,491	3,062,434

[1] Source: FAO (2005).

and means that Vietnam replaced India as the leading producer in 2002. The boost to Vietnam's nut production coincided with the stagnation of the world production. This can be explained by the slow recovery of the African cashew cultivation and the stagnation of the Brazilian production leading to the change in the producers rank.

Presently cashew in India is grown in 730,000 ha with a production of 460,000 mt of raw nuts (Table 9.2). It is one of the world's largest exporters of cashew kernel. Nevertheless, its domestic production is insufficient to supply the industrial demand. This is the reason that it has imported raw material from a number of countries in Africa (Mozambique, Tanzania, Guinea-Bissau, Nigeria), Asia (Vietnam, Indonesia), Australia and South and Central America. Some of these places could aggregate value and provide jobs if the nuts are processed instead of exporting them as raw material.

Mozambique has favourable ecological conditions to the development of this crop. Until the mid-1970s, it was the first cashew world producer with 210,000 mt in 1973. In 1975, the production was severely reduced to 15,000 mt due to its political problems. Due to this problem in Mozambique, the nut production in Tanzania presented a notable development until the year 1974, when it produced 140,000 mt. From this year on its production decreased. However, it increased from 2000 on, reaching 123,000 mt in 2004 (Table 9.2).

In Indonesia, the cultivated area presented a substantial increase in the 1980s reaching 90,000 mt in 2004, in an area of 260,000 ha. In Nigeria, despite the increase of the cultivated area since 1999, the production remained stagnant at 186,000 mt.

In Brazil, approximately 95% of the crop is produced in the three states of the Northeastern region: Ceará, Piaui and Rio Grande do Norte, the poorest region, characterised by having more than half the area under semi-arid conditions. Cashew nut processing industry in these areas plays an important socio-economic role in the urban and rural development. Over the past few decades the cashew nut agribusiness has registered a remarkable increment.

Presently, there are 700,000 ha and an industrial park capable of processing 280,000 mt of nuts annually. The importance of the cashew industry is evidenced by the export revenue to the tune of 140–160 million dollars annually, along with providing thousands of direct and indirect jobs in all activities within the agro industrial chain (Barros et al. 2002; Leite and Paula Pessoa 2002).

In spite of the socio-economic importance of this agri-business for a poor region, where the opportunities for reducing poverty are scarce, the cashew crop is characterised by the low usage of technology. Consequently, the productivity of the nut fell from 635 kg/ha in the early 1970s to 302 kg/ha during the 2002/2003 yield (Ibge 2004), affecting the productivity and export revenue (Paula Pessoa and Leite 1998). So it is fundamental to increase substantially the yield and productivity to make the business more profitable and attractive for new entrepreneurs.

There are technologies capable of increasing production and productivity. So the crop expansion in traditional and in new potential areas as well as the approach in large stretches of unused marginal farmland should be encouraged. The most remarkable technology for the cashew crop is the selected dwarf clones because of

Table 9.2 Evolution of the world cashew nut production

	Year[1]									
Countries	1970	1980	1990	1995	1999	2000	2001	2002	2003	2004
Brazil	20,309	75,000	107,664	185,229	145,437	138,608	124,073	164,539	178,396	223,941
India	123,319	180,266	285,590	321,640	460,000	520,000	450,000	460,000	460,000	460,000
Nigeria	25,000	25,000	30,000	95,000	176,000	184,000	185,000	186,000	186,000	186,000
Mozambique	184,000	71,100	22,524	33,423	58,720	57,894	58,000	58,000	58,000	58,000
Tanzania	107,445	41,416	17,060	63,400	106,500	121,200	121,900	123,000	123,000	123,000
Indonesia	n.a.	9,074	29,907	74,995	89,500	84,200	86,200	87,900	90,000	90,000
Vietnam	2,100	5,600	140,000	202,400	164,800	270,400	292,800	515,200	637,200	640,000
Other countries	49,766	56,759	97,433	149,327	247,313	284,291	296,458	300,430	301,033	301,160
World	511,939	464,215	730,178	1,125,414	1,448,270	1,660,593	1,614,431	1,895,069	2,033,629	2,082,101

[1] –Mt = metric tons. Source: FAO (2005).

the acceptance compared to other technologies. The main impact of these clones initially was to raise the productivity from 300 kg/ha to 1,000 kg/ha of nuts and 9,000 kg/ha of cashew apples in non-irrigated land. Later, it led to the first experiences in irrigation that resulted in as much as 3,800 kg/ha of nuts and over 34,000 kg/ha of cashew apples (Oliveira 2002). Experimentally, the last released dwarf clone produced 1,500 kg/ha of nuts in the sixth year under non-irrigated conditions (Barros et al. 2000). The use of selected clones has been changing the present situation of the cashew agri-business in Brazil.

On the other hand, utilisation of cashew apple (also termed peduncle or false fruit) is gradually giving rise to an important segment of cashew agro industry with a number of industrialised products. There are over 30 products, namely, concentrated juice, sweets, soda and pure, clarified and pasteurised cashew juice called "cajuína", popular in the Ceará State. The cashew apple represents approximately 90% of the fruit weight but only 5–8% of the production has been industrialised. It is necessary to increase its utilisation to aggregate value and profitability. Sales are gradually increasing and new markets are being opened for some of the apple products. In addition, the quality of apple from specific clones has resulted in a surprising increase in the consumption. Post-harvest experiments resulted in conservation of apple up to 20 days, making feasible the opening of new and important markets far from the origin of production and tradition.

9.2 Origin and Domestication

There are two centres of diversity for the genus *Anacardium* in Brazil itself: one in the Amazon region, in the lowland moist, gallery and dry forests and the savannah-like vegetation called "cerrado"; and the other in the central uplands characterised by the cerrado. However, the greatest diversity of the cultivated species, *A. occidentale*, is the "restinga" (sandbank) vegetation, a low dense forest of the sandy soils in the coastal northeast far from the two centres of diversity of the genus (Barros and Crisóstomo 1995). Cashew is now distributed over most of the tropical areas of the world, from 27 °N in southern Florida, to 28 °S in South Africa (Frota and Parente 1995).

The interpretations about the cashew tree origin are supported by circumstantial evidences, such as early bibliographical references, geographical distribution, ecological patterns of distribution, genetic diversity, popular tradition of use and number of products associated with the people background. From these, it is reasonable to accept that Brazil – or at least the northern part of South America and Central America – is the centre of origin of the species. Another evidence is the name caju, which means nut. It came from the Tupi (the native idiom) word "acâ-yu" (acâ – pome; yú – yellow). The words caju and cajueiro (cashew tree) are common in the Brazilian toponymy. This fact has supported the theory of the Brazilian origin. In addition, the cashew denomination in other languages derives from this native name (its pronunciation and/or meaning).

The cashew tree adapts to a relatively wide range of ecological habitats but the greatest concentration of plants is in the coastal zone and transitions up to 15 °S and 15 °N. However, in several parts of that zone, the species has not been well adaptable. Since cashew tree is sensitive to low temperature and frost, the crop has not been recommended for altitudes over 600 m (Frota and Parente 1995) unless the latitude and the market opportunity makes the crop attractive.

The domestication stages and the genetic improvement of the cashew tree in Brazil can be associated with five periods (Paiva et al. 1997). First, the period of the discovery of cashew plants with edible fruits for consumption as fresh fruit and/or preparation of beverages by the aboriginal South Americans. European explorers found cashew was part of the local cuisine. The earliest documents describing cashew exploitation and consumption date from the seventeenth century (Marcgrave 1942).

The second period spans from the 1940s to the 1950s when the extraction of cashew nut shell liquid (CNSL) and the manufacture of a number of cashew apple-based products began. Pacajus Experimental Station (Ceará state) initiated the research. The first activities were towards the collection of plants from natural populations along the coast of the northeast region.

In the third period, from the 1960s to the 1970s, first commercial plantations were established as a result of a government policy for the cashew industry towards poverty alleviation in the northeast region. All plantations were established by directly planting the seed following 10 m × 10 m or 15 m × 15 m spacing. The only criteria used for the seed choice were the nut weight, i.e., around 9 g. At the time, the main objective of the research was to identify plants with high production and nuts weighing more than 9 g. Seeds from the selected plants were distributed for the new plantations.

In the fourth period, from mid-1970s to the 1980s, selected clones of both common and precocious dwarf types were evaluated and the option was for the latter tree. Since then, selection for dwarf types gained importance. As a result, the CCP 06, CCP 76, CCP 09 and CCP 1001 clones were released for commercial purposes. These were the first Brazilian cashew selections and the result was a jump in the nut productivity from 379.4 kg/ha (average of the period 1958–1995) to an average of over 1,000 kg/ha in orchards established with these clones. The CCP 76 is still preferred by the growers whose business is the fruit market. However, CCP 09 has shown better performance under irrigated conditions.

On the other hand, the genetic base of the collection utilised was too narrow and limited in number that the breeding programmes lagged at several points. This has led to difficulties to new selections and increased risks in terms of genetic vulnerability.

The fifth and ongoing period has been characterised by attempts strictly towards demands of cashew crop in the new century, emphasising irrigation, drought and disease tolerance and apple fresh consumption namely, (a) dwarf type tolerant to biotic and abiotic stresses; (b) cashew apple with requirements to the fresh fruit market; (c) nut easily detachable from the apple and a percentage of kernel/nut $\geq$ 28%; and (d) easy peeling kernel weighing $\geq$ 2.5g that is split-resistant to the shelling process.

9.3 Botany

9.3.1 Taxonomy

Cashew belongs to the family Anacardiaceae. The number of species and genera has been well defined. It has been cited ranging from 400 to 600 and 60 to 74, respectively (Barros 1991). Other commercially exploited species of Anacardiaceae include mango (*Mangifera indica*), one of the most important tropical fruits in traded volume, and pistachio (*Pistacia vera*). The genus Spondias has also a number of well known and appreciated fruit species in South and Central America, such as 'umbu' (*S. tuberosa*): native to the Brazilian Northeast semi-arid region, 'cajá' (*S. mombim*): largely used by the ice cream industry, 'cirigüela' (*S. purpurea*) and 'cajá-manga' (*S. cytherea*). All these can be both industrialised and consumed as fresh fruit (Barros 1991).

Through the classical taxonomy, 21 species were recognized (Table 9.3) (Barros 1995). These were subsequently reduced to nine species by numerical taxonomy (Mitchell and Mori 1987). *Anacardium occidentale* L. is the only cultivated species in the genus and the most widely dispersed (Johnson 1973; Ohler 1979; Mitchell and Mori 1987). It is important to emphasise that its natural dispersion pattern can be confounded by the dispersion through cultivation. Thus, while the diversity of the genus *Anacardium* is greatest in the Amazon basin and in the central uplands, the highest degree of diversification of the cultivated species is observed in some

Table 9.3 Species of *Anacardium* described by classic systematic botany (Johnson 1973)

Species	Locale of occurrence
Anacardium brasiliense Barb. Rodr.	Brazil
A. curatellaefolium St. Hil	Brazil
A. encardium Noronha	Malaysia
A. giganteum Hancock ex. Engl.	Brazil
A. humile St. Hil	Brazil
A. mediterranneum Vell. Fl. Flum	Brazil
A. nanum St. Hil	Brazil
A. occidentale Linn.	Brazil
A. rhinocarpus D. C. Prod.	Brazil
A. spruceanum Benth Ex. Engl.	Brazil
A. microsepalum Loesn	Amazon region
A. corymbosum Barb. Rodr.	Brazil
A. excelsum Skeels	Brazil
A. parvifolium Ducke	Amazon region
A. amilcarianum Machado	Brazil
A. kuhlmannianum Machado	Brazil
A. negrense Pires & Fro'es	Brazil
A. rondonianum Machado	Brazil
A. tenuifolium Ducke.	Brazil
A. microcarpum Ducke.	Amazon region
A. othonianum Rizz.	Brazil

environments of Brazilian Northeast. This suggests that the speciation and origin of cashew crop is related to this region (Barros 1995).

9.3.2 Morphology

Cashew is an evergreen tree with low-branching and medium-size canopy. On an average, the plant reaches 5–8 m high and 12–14 m wide, though some plants with 15 m height and 20 m width have been observed. The leaves are single, alternate, oblong, leathery and smooth and measure from 10 to 20 cm length and from 6 to 12 cm width. Leaf size is variable due to the species natural genetic diversity.

The root system of a complete grown plant consists of a taproot surrounded by a network of lateral secondary roots most of which (91%) lie on the15–32 cm depth soil layer and have rootlets. In plants up to six years old, the ratio between lateral root and canopy width is close to 2:1. In completely developed plants, these lateral roots radiate horizontally as far as 20 m from the trunk. The plant growth cycle usually alternates a vegetative and a reproductive phase. Because these phases are regulated by both genetic and environmental factors, growth patterns vary among individuals.

The inflorescence is a terminal panicle with both male flowers (Fig. 9.1) and hermaphrodite or perfect flowers (Fig. 9.2). Hence, cashew is an andromonoecius species. The number of panicles per plant, flowers per panicle and the distribution of the two types of flowers in each panicle vary enormously (Rao and Hissan 1957; Pillai and Pillai 1975; Damodaran et al. 1966; Pinheiro et al. 1993). Genetic and environmental factors are responsible for these variations. The flowering is gradual, so each panicle may last up to three months, resulting in a continuous fruit setting. The duration of this phase depends on the genotype and environmental conditions, specially the rainy season. The practical importance of this characteristic is related to

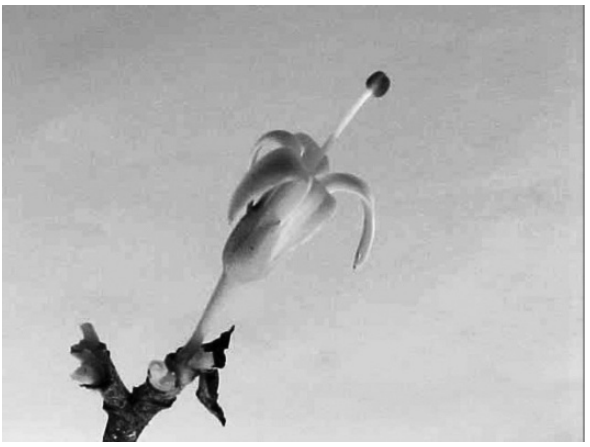

Fig. 9.1 Male (stamened) cashew flower (*See* Color Insert)

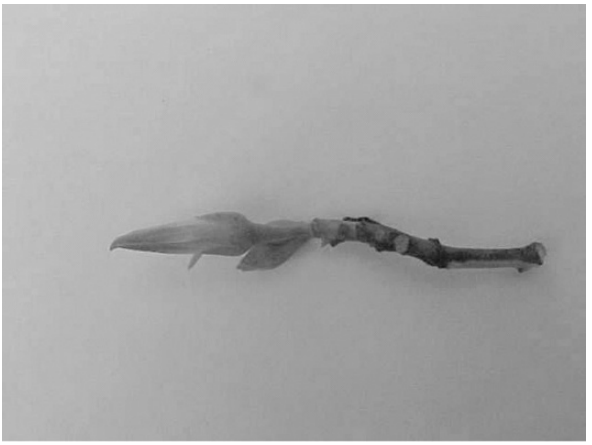

Fig. 9.2 Hermaphrodite (perfect) cashew flower (*See* Color Insert)

the number of employees involved in the harvest. It is also beneficial to the growers involved in fresh fruit business. The flower has 5 sepals, 5 petals, a simple ovary (rudimentary in male flowers), and 7–15 stamens, though most often 10 (1 large and 9 small). The stigma of hermaphrodite flowers is usually longer than the stamens making self-fertilisation within the same flower more difficult and possibly serving as an evolutionary mechanism in favour of cross-pollination. However, the geitonogamy (pollination between flowers of the same genotype) is also expected to be possible due to the absence of a self-incompatibility system.

Flowering phase lasting depends on the environmental conditions and the genotype. In the coastal region and transition zones of Northeastern Brazil (around 6 °S latitude and up to 100 m altitude), it lasts 4–6 months in the common type (from July/August to December/January) and 6–8 months (from June/July to January/February) in the precocious dwarf type (Barros et al. 1984; Barros 1988; Freitas 1994.). Under special conditions some genotypes can flourish throughout the year.

Anthesis starts at about 6 a.m. and continues till 4 p.m. Hermaphrodite flowers open mostly between 9 and 11 a.m., with little variation. The viability of the pollen is usually high and the stigma becomes receptive one day prior to the unfolding of the flower and the receptivity lasts for two days. The strong smell of the cashew flower attracts pollinating insects especially the bee (Apis mellifera). Cashew pollen is not easily carried by the wind (Paulino 1992; Freitas 1994; Freitas and Paxton 1996). Freitas and Paxton (1996) observed that only pollen from the largest stamens, belonging to both male and hermaphrodite flowers, are viable and participate in the reproductive process. Pollen grains from the smaller stamens are not viable.

The flowers are small with short pedicels and yellow-white in colour, changing to reddish after the fertilisation. They are disposed in stalked and outbranching terminal panicles supported by bracts. The reproductive system of the species is predominantly allogamous, favouring cross-fertilisation. Nevertheless, the simultaneous

presence and blossoming of the two types of flowers on the same plant and within the same panicle also favours self-pollination and, consequently, endogamy. No system of auto-incompatibility has so far been observed among the species of the genus.

The breeding system may allow pollen donation from the same flower resulting in autogamy and selfing. It also allows pollen donation from different flowers of the same and of different plants resulting in allogamy. If the pollen donation is from different flowers of the same plant it results in geitonogamy and selfing. If from flowers of different genotypes, it results in xenogamy and crossing. However, there is no information about the cashew behaviour in relation to these possibilities.

The fruit, the nut, is a kidney-shaped achene consisting of epicarp, mesocarp, endocarp and a kernel wrapped by a peel (testa). The nut weight varies greatly, from less than 3 to 33 g to more. At the industry it averages 8 g. The epicarp is smooth, coriaceous and grey or greyish green; the mesocarp is thick, spongy and features alveolae containing the so-called cashew nut shell liquid (CNSL), a caustic and rich in phenolic compounds. The kernel – the edible part of the nut – is formed by two cotyledons and is covered by a thin membrane (peel). The peeling is one of the main problems in the industrial processing as it is very difficult to remove in as much as 20% of the seeds. The hypertrophied pedicel develops into the cashew apple or 'false fruit'; it is fleshy and juicy and varies in size, weight, shape, texture, colour and chemical compounds. The false fruit is often referred to simply as 'cashew', although the term may also be applied to the cashew apple and nut together.

The nut grows slowly in the first stages and then accelerates to attain its maximum size by 28 days. In the following days the nut size reduces as the maturation evolves and becomes visibly smaller (Damodaran et al. 1966). The cashew apple, on the other hand, reaches its maximum size when the maturation is complete, without any further change in size.

9.3.3 Botanical Types

Barros (1988) classifies the natural diversity of cashew tree in two groups: common type and dwarf type (Table 9.4). The common type, the most diffused, has 8–15 m of plant height and up to 20 m of canopy width. The branch distribution and the canopy frame vary greatly, ranging from erect and compact to sprawling. The productive capacity also varies considerably, from a few fruits to an unknown record of nuts per year. The nut weight ranges from less than 3 to 33 g and cashew apple weight ranges from 20 g to over 500 g. The production stabilises after 8 years, frequently at 12–14 years.

The precocious dwarf cashew, also known as the "six-month variety", is 4 m high with a homogeneous 5–6.5 m wide canopy. The flowering starts 6–18 months after planting. Among natural populations the nut weight ranges from less than 3 to 10 g and the apple weight from 20 to 160 g. The individual productive capacity is also smaller: the highest yield observed so far was 43 kg of nuts (Barros 1988). The variability for the main agronomic traits is lesser than that observed in the common type.

Table 9.4 Main agronomic differences between the common and the precocious dwarf types

Common type	Dwarf type
1. Origin – Northeastern Brazil	1. Probable origin – Amazon region
2. High stature (8–15 m)	2. Low stature (< 4m)
3. Canopy width (10–20 m)	3. Canopy width (5–6.5 m)
4. First flowering (3–5 years)	4. First flowering (6–18 months)
5. Hermaphrodite flowers (7.9%)	5. Hermaphrodite flowers (3.9%)
6. Nut weight (3–33 g)	6. Nut weight (3–10 g)
7. Apple weight (20–500 g)	7. Apple weight (20–160 g)
8. Production of nuts/plant/season (< 1 to > 230 kg)	8. Production of nuts/plant/season (up to 43 kg)

9.4 Reproductive Biology

9.4.1 Hand-Pollination

Cashew genetic improvement is based mainly on crosses among selected genotypes, and evaluation of hybrids and half-sib progenies derived from the hybrids. The pollination technique consists of:

a. Flower preparation – emasculating the anthers of male and hermaphrodite flowers of the female genitor (Fig. 9.3);
b. Pollen collection – transferring male flowers from the inflorescences of the male genitor early in the morning to Petri dishes with moistened cotton;
c. Pollination – between 9 and 11a.m. (when the hermaphrodite flowers are unfolding and the time is appropriate for anther dehiscence), shaking the collected

Fig. 9.3 Emasculation of male anthers during preparation of inflorescence for pollination (*See* Color Insert)

Fig. 9.4 Cashew pollination (*See* Color Insert)

flowers above the stigma of the recipient flower, loosening the grains and making them to adhere to the surface (Fig. 9.4);

d. Evaluation – taken after one week (Fig. 9.5). Table 9.5 highlights the success rates of controlled pollination between cashew clones CCP 76 and CCP 1001. The rates were relatively high (> 60%) for self-fertilised plants and for the CCP 76 × CCP 1001 hybrid (Paiva et al. 1998). On the other hand, when the CCP 1001 clone was the female genitor, reciprocal crosses resulted in less than 40% success. The cause has been attributed to the influence of cytoplasmic inheritance upon pollination efficiency

Fig. 9.5 Protection of pollinated flower

Table 9.5 Success rates for controlled cashew pollination in the Pacajus Experimental Field, Embrapa

Clones	No. flowers pollinated	Pollination rate (%)	Fruit set (%)	Seed germination (%)
Controlled pollination				
1. Self-fertilisation				
CCP 76	808	63.1	4.7	84.0
CCP 1001	167	61.1	4.2	63.0
2. Crossing				
CCP 76 × CCP 1001	796	64.6	8.0	86.0
CCP 1001 × CCP 76	644	39.1	14.6	81.0
3. Free pollination				
CCP 76				94.0
CCP 1001				86.0

9.5 Genetic Resources

Because of its potential for generating job and income in areas with few economically feasible alternatives, the cashew has been introduced into several agro-ecosystems. This continuous expansion of cultivated areas calls for clones adapted to new environments. Therefore, any comprehensive improvement programme for developing clones for new agro-ecosystems needs genetic diversity.

The existence of only a few commercial clones, selected from a germplasm with a narrow genetic base and evaluated in one environment, is a risk to genetic vulnerability (Almeida et al. 1992, 1993; Barros et al. 1993, 2000). The solution is in creating and selecting new genotypes to add and/or replace dwarf clones now being cultivated.

Cashew is predominantly allogamous, highly heterozygotic and hence demands large samples to represent natural variability. The conservation of genetic resources, though costly, is indispensable. Hence, it is rarely possible to adopt the traditional conservation schemes: collection, conservation, characterisation, evaluation and use. Evaluation and characterisation become increasingly difficult in the Active Cashew Germplasm Bank (ACGB). In addition, regardless of their respective importance, both collection and conservation are costly research activities with little short-term economic returns (Paiva et al. 2003b) (Fig. 9.6).

The cashew germplasm collection presently consists of 621 accessions (Table 9.6), of which 565 are of the cultivated species *Anacardium occidentale* L., and the remainder are from the savanna regions, such as *A. microcarpum* Ducke, *A. othonianum* Rizz, *A. humile* and *Anacardium* sp. About 70% of the accessions are from Ceará state that reduces the representativeness of the conserved germplasm. Forty accessions of apparently drought tolerant precocious dwarf type were introduced and 75 selected plants selected from the experiments running to evaluate half-sib progenies of dwarf type for different characteristics were cloned and added in the ACGB as accessions. The collection of common cashew was resumed in 2001 with the addition of 73 accessions: 20 asexually propagated and

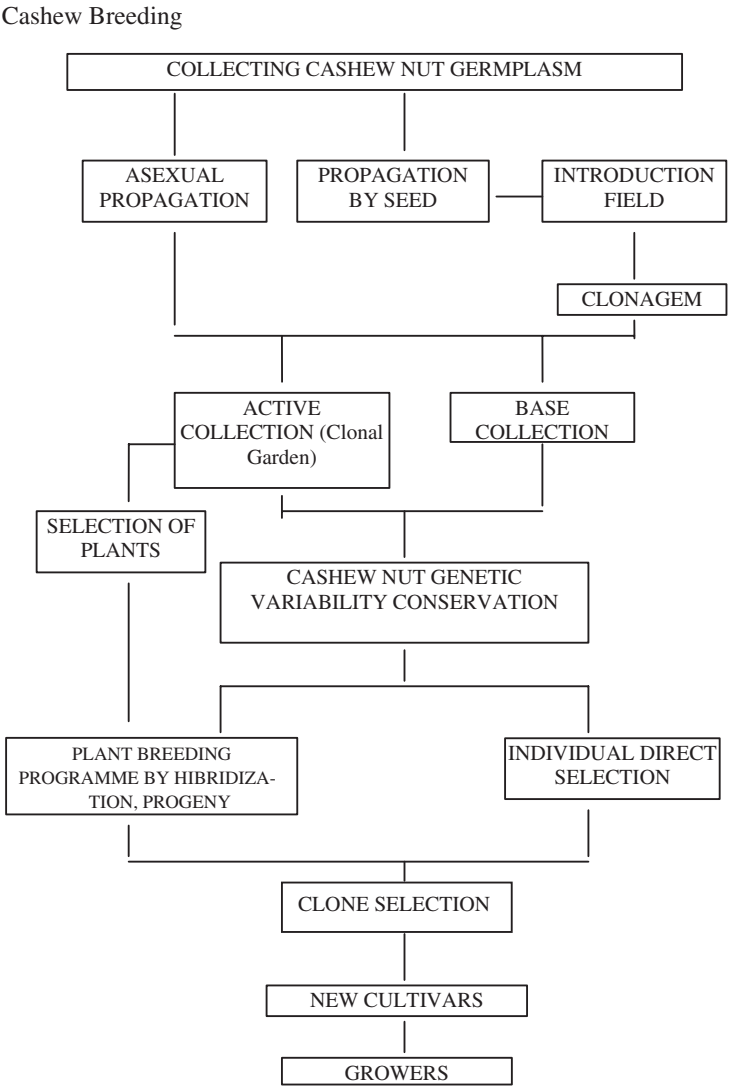

Fig. 9.6 Active cashew germplasm bank : collection, conservation, evaluation and use of genetic variability

53 by seedlings. The total for 2002 was 52 accessions: 26 by seedlings and 26 asexually propagated.

As cashew is primarily a cross-pollinated crop, it is highly heterozygous. Significant segregation has resulted in large variations in the populations. In India, collecting and conserving the germplasm is an important activity in the crop improvement programme. Cashew germplasm collection and conservation has been carried out at the National Research Centre for Cashew (NRCC), Puttur (Karnataka) since

Table 9.6 Accessions introduced in the Embrapa cashew active germplasm bank (CAGB) from 1956 to 2002

Year	Origin	Species	No. of accesses	No. of plants	Propagation mode
1956/1969	Pacajus, CE1	Common type (*Anacardium occidentale* L.)	124	3.023	Seed
1979	Russas, CE	“	16	40	Asexually
1979	Cascavel, CE	“	9	25	Asexually
1979	Aracoiaba, CE	“	16	42	Asexually
1979	Aracati, CE	“	15	27	Asexually
1979	Trairi, CE	“	16	30	Asexually
1985	Pará, PA2	“	5	24	Seed
1985	Mato Grosso, MT3	“	3	9	Seed
1985	Bahia, BA4	“	1	2	Seed
1973	Valinhos, SP5	“	1	10	Seed
1973	Índia	“	1	9	Seed
1975	Índia	“	09	77	Seed
1973	Venezuela	“	1	09	Seed
1988	Pio IX, PI6	“	26	26	Seed
1988	Icapui, CE	“	21	21	Seed
1995	Itau, RN7	“	4	12	Seed
1995	Severiano Melo, RN	“	6	18	Seed
1995	Serra do mel, RN	“	6	18	Seed
1995	Pacajus, CE	“	3	3	Seed
1995	Apodi, RN	“	2	6	Seed
1956	Maranguape, CE	Dwarf type (*Anacardium occidentale* L. var. nanum)	08	21	Seed
1994	São Luiz do Curu, CE	Common type (*Anacardium occidentale* L.)	19	193	Asexually
1994	Pio IX, PI	“	21	170	Asexually
1995	Pio IX, PI	“	20	60	Asexually
1995	Pacajus, CE	“	12	30	Asexually
1996	Pacajus, CE	“	75	75	Asexually
1964	Brazilian Northeast region	Non-cultivated Species with very small fruit and red apple (*Anacardium microcarpum*)	1	18	Asexually
1964	Brazilian Northeast region	Non-cultivated species with very small fruit and red apple (*Anacardium microcarpum*)	1	31	Seed
	Brazilian Northeast region	Non-cultivated species with very small fruit and yellow apple (*Anacardium microcarpum*)	1	9	Seed
1975	Roraima, RR8	*Anacardium* sp.	18	47	Seed

Table 9.6 (continued)

Year	Origin	Species	No. of accesses	No. of plants	Propagation mode
1985	Roraima, RR	*Anacardium* sp.	1	10	Seed
1977	Camocim, CE	*Anacardium* sp.	1	5	Seed
1985	Piauí and Ceará	*Anacardium* sp.	3	23	Seed
	'Unknown origin'	*Anacardium* sp.	1	5	Seed
1984	Goiás, GO9	*Anacardium* othonianum	1	1	Seed
1985	Goiás, GO	*Anacardium othonianum*	18	96	Seed
	'Unknown origin'	*Anacardium othonianum*	1	15	Seed
1985	Goiás, GO	*Anacardium humile*	8	45	Seed
	'Unknown origin'	Cajueiro-do-cerrado	1	7	Seed
2001	Western Ceara (Camocim, Jijoca, Granja e Chaval)	Common type (*Anacardium occidentale* L.)	21	378	Seed
2001	Western Ceara (Camocim, Jijoca, Granja e Chaval)	Common type (*Anacardium occidentale* L.)	20	100	Asexually
2001	Beberibe, CE	Common type (*Anacardium occidentale* L.)	32	576	Seed
2002	Pio IX, PI	Common type (*Anacardium occidentale* L.)	15	270	Seed
2002	Pio IX, PI	Common type (*Anacardium occidentale* L.)	15	75	Asexually
2002	Chorozinho, CE	Common type (*Anacardium occidentale* L.)	11	198	Seed
2002	Chorozinho, CE	Common type (*Anacardium occidentale* L.)	11	55	Asexually
		Total	621	5.944	

CE – Ceará State; PA – Pará State; MT – Mato Grosso State; BA – Bahia State; SP – São Paulo State; PI – Piaui State; RN – Rio Grande do Norte State; RR – Roraima State; GO – Goiás State.

1986. The National Cashew Gene Bank (NCGB) has been established at Puttur (Karnataka). A total of 419 cloned accessions has so far been collected and conserved in the NCGB. In view of the fast replacement of the presently available local germplasm material (diverse types/landraces) with the released high yielding varieties, the collecting and conservation programme should receive priority attention. The majority of the cashew plantations raised by the Forest Department and Karnataka Cashew Development Corporation Limited (KCDC) are of seedling origin and some of the plantations are as old as 30–40 years (Bhaskara Rao and Swamy 2002).

9.6 Breeding Objectives

Because of the low productivity, yield is the main objective of the cashew improvement. This is reinforced by the nut price at the producer level and the fact that there is no differentiated price per nut weight, even though the kernel price is differentiated

in the market. Therefore, selection primarily targets dwarf or dwarf-like plant (to make manual harvesting easier) yielding over 1.5 t of cashew nuts/ha under rainfed conditions (Barros et al. 2000). Drought tolerance is a relevant attribute in the evaluation of genotypes. Irrigation is becoming a reality in restricted areas, so it is necessary that genotypes yielding over 3.8 t/ha are possible to achieve with currently available clones (Oliveira 2002). Research is also being carried out on salt and aluminum tolerance as there is a huge area in cerrado.

Apart from production, selection parameters for both irrigated and non-irrigated crop are prioritised as: (a) cashew apple with requirements to the fresh fruit market – red or reddish colour, texture, shelf life, consistency, tannin content and taste; (b) nut easily detached from the apple and the percentage of kernel/nut weight $\geq$ 28%; (c) kernel easy peeling, weight $\geq$ 2.5g and split resistant to the shelling process.

The variability of available germplasm suggests that the objectives of breeding are attainable once positive results are already obtained for nut yield and apple quality (Barros et al. 2000; Moura 1998; Oliveira et al. 1998; Paiva et al. 1998).

Pests and diseases in all phases of the plant development also affect the yield and must be taken into account in the breeding programmes. Bleicher et al. (1993) presented a list of 97 species of insects and 5 of mites associated with cashew in Brazil. Of these, only a few are considered major pests and its importance vary in time and space. Considering their geographic distribution and economic importance, the most important pests to the cashew crop are the borer (*Antistarcha binocularis*), the cotton aphid (Aphis gossypii), the cashew nut moth (*Anacampsis* sp.), the thrip (Selenothrips rubrocinctus), the white fly (*Aleurodicus cocois*) and some coleopterous borers of the genera Marshallius and Apate.

The diseases also play a significantly negative role in the cashew yield and fruit quality. Major leaf diseases are anthracnose (*Colletotrichum gloeosporioides*) and black mold (*Pilgeriella anacardii*). These are currently responsible for great losses in both nut yield and nut and apple quality. And gummosis, caused by the fungus *Lasiodiplodia theobromae* (Pat.) Grif., has become increasingly important in semiarid areas in Northeastern Brazil (Cardoso et al. 1998).

The use of resistant clones represents an economical, ecological and safe form of pest and disease management. It also provides a better usage of the genetic variability; both naturally found and that generated from the progeny obtained in the breeding programmes.

9.7 Breeding Methodologies

The reproductive behaviour of a species has direct bearing on the breeding methodologies applied for its improvement (Allard 1971). As a cross-pollinated species, cashew needs to be dealt specifically with emphasis on asexual/vegetative propagation.

The commonly used breeding procedures for a species with asexual propagation are germplasm introduction, clonal selection and hybridisation. Other

unconventional methods such as induction of mutations, polyploidy and biotechnologies have been used with limited success.

Apart from plant introduction, the cashew may also be bred through the following processes: (a) population improvement – by evaluating progenies from selected plants with desired characteristics; (b) clonal improvement – through selecting and evaluating cloned plants with desired attributes; (c) population improvement followed by clonal selection – through obtaining clones in families derived from selected plants and/or populations in the area of dispersion of the species.

The strategies, schemes and methods, as well as expected progress and problems related to, will be discussed here.

9.7.1 Plant Introduction

Plant introduction has been an important source of genetic resource for commercial purposes. The crop in other than the natural ecosystem has relied on the precocious dwarf type, especially the clones CCP 09 and CCP 76. The genetic material should be as diversified as possible not only because these clones may not be adaptable to certain ecosystems, but also to avoid creating a situation of genetic vulnerability as a result of genotypic uniformity in the areas of exploitation.

9.7.2 Polycross Method

Polycross is a method for improving allogamous species, especially those with small hermaphrodite flowers (Vello and Vencosvky 1982). This method yields results similar to those obtained through bi-parental crosses, in spite of the possibility of self-fertilisation effects once there is no control of one of the parents. It is also less costly because of the reduced requirements towards skilled labour. Also, greater number of genotypes can be dealt with leading to wider genetic base. As the number of parents increases, the relative selection rate also increases.

Polycross method was first applied in the cashew breeding at the Pacajus Experimental Station in 1978 aimed at obtaining favourable combinations of dwarfism, precocity and high nut yield and weight (Barros et al. 1984). The procedure was initially as described in the methodology. Eight genotypes were selected for their desirable traits and 64 plants (8 of each genotype) were arranged in a way to favour chance for crosses among all genotypes. This was finally reduced to 49 plants (with 7 plants per genotype) due to the elimination of one precocious dwarf type. In the third year, all plants produced enough seed to perform the ‘polycrossed’ progenies. The analysis was done by considering 49 treatments. This modification was made in order to increase the number of treatments. Moreover, cashew tree can be asexually propagated, cloning desirable genotypes at any stage of the process (Barros et al. 1993). In the following years, the progenies were evaluated and one genotype resulted in the clone EMBRAPA 51 (Barros et al. 2000).

9.7.3 Population Improvement

Population improvement makes it possible to obtain the best lineages for hybrid combinations in allogamous species by increasing the frequency of favourable genes (Paterniani and Miranda Filho 1978). Effective success in population improvement depends fundamentally on the presence of genetic variability in the original population. However, other factors are also important, such as: the method of selection, the degree of statistic precision, the correct interpretation of environmental effects, the estimation of genotype × environment and genotype × year interactions, the identification of pleiotropic effects and genetic and phenotypic correlation among characters (Paterniani and Miranda Filho 1978; Vencovsky 1978).

The processes of selection utilised in cashew tree population improvement are described below:

9.7.3.1 Individual Phenotype Selection

In cashew nut, yield factors, nut and kernel weight, plant size and precocity have been the main traits in all programmes. Nut production is still the most important trait for the newly selected clones, so also the characteristics such as drought, toxic aluminium tolerance and resistance to anthracnose.

The practical advantage of this method is that genotypes can be selected independent of the environmental factors like irrigation, fertiliser, pruning, etc. The main shortcoming of this methodology is the real possibility of loss of genotypes with potentialities not expressed in the environment in which the selection is made because of adverse conditions as well as inadequate evaluation. Nor is there any control of the male genitor. So selected plants are pollinated with a random mixture of pollen that ensures chances for crosses involving more closely related genotypes or those preferred by the pollinating agents. Parent control occurs only on the female side. The lack of controlling both the environment and the male genitor is minimised or becomes negligible when clonal selection or hybrid production follows individual selection.

9.7.3.2 Progeny Test

Progeny-based selection is always more efficient than selection based on individual phenotypes alone as in mass or phenotypic selection, because not only selected individuals but also their descendents are evaluated (Paterniani and Miranda Filho 1978). Several selection processes use the progeny test to evaluate genotypes, each process exploring a different part of the genetic variation. In theory all processes are applicable to cashew breeding, since the cashew tree is an allogamous species with a great number of descendants per generation.

In practice, however, these methods are almost impossible to apply because of the short time to carry out the improvement activities required by most crop development programmes. Vencovsky (1978) offers a comprehensive representation of the different schemes including an expression for the selection gain in relation to

the time for each cycle of selection and the required area for evaluating a population under improvement. In addition, the association between the advantage of the vegetative multiplication at any stage with the disadvantage of seed-based commercial cashew crop has led to adaptations of the known methods in order to reduce time to obtain new clones.

9.7.4 Hybridisation

Hybridisation between individuals or populations with different combinations of adaptive genes can expand the gene pool in relation to genes with different adaptive values, as long as the hybrids are able to produce segregating progeny in future generations (Stebbins 1974). Moreover, as a breeding method, hybridisation makes it possible to join in one individual the economically desirable characteristics from different genotypes.

The cross of cashew clones results in segregating progenies because each individual is essentially a hybrid. Consequently, the individuals originated from the crosses or hybrids are in fact a set similar to the F2 generation. Segregation is more intense in this phase and selection should be initiated in order to keep clone the best genotypes. However, selection intensity should be carefully observed to avoid drastic discard in the number of individuals and loss of variability.

Seed-propagated plants allow evaluation of crosses through the methodology of general and specific combining ability. Cavalcanti et al. (1997) using this methodology observed differences among genotypes and their crosses in relation to early nut yield. Moreover, Cavalcanti et al. (2000) detected significant discrepancies in relation to the traits plant height, canopy diameter, number of nuts, nut yield, nut weight and weight of kernel.

The efficiency of artificial hybridisation between precocious dwarf plants and common type plants, carried out at the Pacajus Experimental Station, was 16.5% (range 8.9–28.9%) of fructification (Table 9.7). The average crosses among dwarf genotypes (17.4%) was slightly higher than crosses between dwarf and common genotypes (15%). This difference cannot be considered significant.

Highly heterozygous hybrids typically exhibit enhanced vigour, a feature sometimes referred to as heterosis (Winter et al. 1998). Indeed, this phenomena has been observed to influence attempts to improve a wide range of agronomic traits in cashew. Damodaran (1975) noted evidence of hybrid vigour with increments of up to 153% in the nut yield relative to plants derived from outcrossed pollinations. Similarly, Manoj and George (1993) cited the occurrence of heterosis in cashew hybrids in relation to nut yield, nut weight and weight of kernel. In a later work, Cavalcanti et al. (2000) evaluated crosses between common and dwarf types and detected average increases in plant height (20%), canopy diameter (32%), number of nuts (121%), nut yield (192%), nut weight (15%) and weight of kernel (19%). For crosses among dwarf types, Cavalcanti et al. (2003) identified average increases of 12, 19, 98, and 97% in relation to the traits plant height, canopy diameter, number of

Table 9.7 Efficiency of artificial hybridisation in dwarf cashew tree and dwarf and common type genotypes in the Pacajus Experimental Station, Ceará, Brazil

Crosses	Pollinated flowers	Harvested fruit	Frutification (%)
Dwarf × Dwarf			
CCP 09 × CCP 06	114	33	28.9
CCP 09 × CCP 76	170	23	13.5
CCP 09 × CCP 1001	112	19	17.0
CCP 09 × C1P 3	97	21	21.6
CCP 09 × 399	192	28	14.6
CCP 1001 × CCP 06	167	31	18.6
CCP 1001 × 399	149	20	13.4
Subtotal	1, 220	212	17.4
Dwarf × Common			
CCP 09 × Matrix 07	228	25	11.0
CCP 09 × Matrix 12	172	29	16.9
CCP 09 × Matrix 77	126	18	14.3
CCP 09 × Matrix 96	112	10	8.9
CCP 09 × B. Ton.	141	35	24.8
Subtotal	779	117	15.0
Total	1, 999	329	16.5

Source: database EMBRAPA/CNPAT.

nuts per plant and nut yield, respectively. All these works were said to be indicative of heterosis and have led the cashew breeding programme to concentrate efforts for using the hybridisation strategy in order to generate superior hybrids to produce elite clones.

9.7.5 Clone Selection

Clone selection, one of the steps in the breeding of asexually propagated plants, is used after plant introduction and hybridisation. The success of this methodology depends on the availability of superior genotypes. According to Simmonds (1979), breeding of clones is reduced to the crossing of heterozygous clonal parents followed by selection among F1 seedlings and among subsequent vegetative generations. The objective is to identify outstanding genotypes and organise a clonal bulk of them. This method is acceptable considering that even in clonal selection the individual is the result of a cross and therefore can be considered a hybrid. In this case, the crossing of two individuals actually produces F_2-like plants so the seedlings must be highly segregating.

Selection must be as weak as possible at the beginning except for high heritability characters. It may be intensified when the number of individual clones is large enough to reduce the effect of environmental variance. Results are more reliable when selection is based on data from several places and different years. An ideal population size should be found in order to maximise all previously unexplored genetic potential (Simmonds 1979).

Till date, an ideal clone with all desirable attributes has not been recommended for commercial use. All Brazilian cashew orchards using plants of the common type have been established through seedlings. Clones are in demand because of the low yield of common types, the heterogeneity of plants in orchards and the heterogeneity of nuts and apples produced by sexually propagated plants.

Research addressed to common type clones began in the 1970s. The first selection took into account yield, plant height and canopy expansion of 20 clones grafted onto two rootstocks (common and precocious dwarf type). The results were not encouraging as the plants yielded lesser than their parents. In fact, on average, they produced less than 10% of the maximum yield registered by the parents. These results explain why none of these clones were released to producers (Barros et al. 1984). No acceptable explanation was found as to why all research efforts were concentrated on the dwarf type, slowing down the common type improvement. However, the situation changed during 2001 when genotypes with favourable agronomic and economic characteristics were selected in a private farm. As a result, clones from medium-sized plants and nuts with kernels above the minimum weight limit established by the international classification (special large whole (SLW), weight: 2.54 g) were brought under evaluation.

This resume in the use of common type germplasm relies on the genetic variability for nut yield observed in the commercial orchards, all established from seedlings. Thus the theoretical potential yield of orchards is over 3,000 kg of nuts/ha (in a 100 plants/ha density) under rain-fed conditions since it is very easy to find plants producing over 30 kg of nuts/year. The first approach to the precocious dwarf type improvement started in 1965 at the Pacajus Experimental Station. The methodology utilised was individual phenotypic selection for nut yield and weight. Though of limited genetic gain, this methodology made it possible to launch the commercial clones CCP 06 and CCP 76 in 1983, CCP 09 and CCP 1001 in 1987 (Barros et al. 1984; Almeida et al. 1992; Paiva et al. 2002). It gave the beginning of a new era in cashew research.

9.7.5.1 Commercial Clones

From 1965 till date no more than a dozen clones were released. Of these, the following are the most representative:

Clone CCP 06 (Cashew Clone from Pacajus 06 Genotype)

Obtained from the CP 06 genotype, in 1979. This genotype belonged to a batch of precocious dwarf plants collected in Maranguape County (30 km southwest of Fortaleza, the capital of Ceará State) and introduced by seed in 1956 at the Pacajus Experimental Station. The highest yield registered to this genotype was 25 kg of nuts. The clone was evaluated in a low fertility sandy soil without correction, fertilisation or pest control. It was commercially released in 1983. It is a 2.11 m tall and 4.52 m wide canopy tree by the sixth year (Fig. 9.7). These are precocious dwarf type peculiar characteristics. The nut and peeled kernels (including natural

Fig. 9.7 Plant of the cashew clone CCP 06 evaluated under irrigated conditions at the experimental station (*See* Color Insert)

humidity) average weight are, respectively, 6.4 g and the 1.6 g. The percentage of kernel/nut is 24.8% and the split kernel in industrial processing is 9.3%. The yield during the fourth year is 3.5 kg/nut/plant (Barros et al. 1984, 2000). The yellow apple averages 76.5 g (Fig. 9.8). Because the weight of the nut is undesirable for industry use the clone is out of commercial usage, being presently much used as a source of seeds for rootstock.

Clone CCP 09 (Cashew Clone from Pacajus 09 Genotype)

Selected from the CP 09 genotype by the same procedure and from the same germplasm utilised in the former clones, but at another time and experiment. It was released in 1987. The highest yield registered to this genotype was 25 kg of nuts. It is a 2.15 m tall and 4.65 m wide canopy tree by the sixth year (Fig. 9.9). The nut

Fig. 9.8 Peduncle of the cashew clone CCP 06 evaluated under non-irrigated conditions at the experimental station (*See* Color Insert)

Fig. 9.9 Nine-year-old plant of the cashew clone CCP 09 evaluated under irrigated conditions at the experimental station (*See* Color Insert)

and peeled kernels (including natural humidity) average weight are, respectively, 7.7 g and the 2.1 g. The percentage of kernel/nut is 27.7%, the highest among all clones and the split kernel in industrial processing is 9.7%. Of all commercial dwarf clones, this is the most affected by the irregular rainfall due to which yield ranges from 600 to 1,200 kg/ha in non-irrigated crop and 3,800 kg/ha of nuts in irrigated crop. Experimentally it has obtained yields of over 4,500 kg/ha of nuts in irrigated crop. The orange to reddish apple averages 87 g (Fig. 9.10) and is well accepted in the fresh fruit market, the reason being that this clone is the second most cultivated. (Barros et al. 1984, 2000; Oliveira 1999; Paiva et al. 2003a)

Fig. 9.10 Peduncle of the cashew clone CCP 09 evaluated under non-irrigated conditions at the experimental station (*See* Color Insert)

Fig. 9.11 Plant of the cashew clone CCP 76 evaluated under non-irrigated conditions at the Pacajus experimental station (*See* Color Insert)

Clone CCP 76 (Cashew Clone from Pacajus 76 Genotype)

Selected from the CP 76 genotype by the same procedure and from the same germplasm utilised in the clone CCP 06, it was released in 1983. The highest yield registered to this genotype was 22 kg nuts. It is a 2.68 m tall and 4.98 m wide canopy tree by the sixth year (Fig. 9.11). The nut and peeled kernels (including natural humidity) average weight are, respectively, 8.6 g and the 1.8 g. The percentage of kernel/nut is 20.1% and the split kernel in industrial processing is 4.1%. This clone is still the most cultivated and the yield varies according to the environment and technology level adopted in the crop. So yield ranges from 600 to 1,000 kg/ha of nuts in non-irrigated crop at the stabilization period. Experimentally, yield ranges from 600 to 1,200 kg/ha in non-irrigated crop and up to 2,500 kg/ha of nuts in irrigated crop (Barros et al. 1984, 2000; Oliveira 1999; Paiva et al. 2003a). The apple colour is orange and the average weight is 135 g (Fig. 9.12). This clone has been most exploited under irrigation, mainly when the objective is the apple, the most sold in the fresh fruit market. Under irrigation, the weight of the nut, kernel and apple is above average in non-irrigated crop.

Clone CCP 1001 (Cashew Clone from Pacajus 1001 Genotype)

Selected from the CP 1001 genotype by the same procedure and from the same germplasm utilised in the former clone, it was released in 1987. The highest yield registered to this genotype was 43 kg of nuts. It is a 2.78 m tall and 5.04 m wide canopy tree by the sixth year (Fig. 9.13). The nut and peeled kernels (including natural humidity) average weight are, respectively, 7.0 g and the 1.9 g. The percentage of kernel/nut is 28.1%, the highest among all clones, and the split kernel in industrial processing is 9.5%. The yield ranges from 547 to 1,493 kg/ha in non-irrigated crop by the sixth year, depending on the rainy season and orchard management. The

Fig. 9.12 Peduncle of the cashew clone CCP 76 evaluated under non-irrigated conditions at the Paraipaba experimental station (*See* Color Insert)

Fig. 9.13 Nine year-old plant of the cashew clone CCP 1001 evaluated under irrigated conditions at the Paraipaba experimental station (*See* Color Insert)

orange to reddish apple averages 85 g (Fig. 9.14) also can be exploited in the fresh fruit market (Barros et al. 1984, 2000; Oliveira 1999; Paiva et al. 2003). Under irrigation plants grow more than the others do and there is no proportional nut production. Another remarkable characteristic of this genotype is the high variation in the nut weight into one panicle and among panicles into a plant. So, it is possible to find nuts weight ranging from 5 g to 10 g of the same plant.

Embrapa 50

This clone was originated from the individual selection phase of the progenies evaluation in a population-breeding programme followed by clonal evaluation. This particular progeny came from a crossing between the clone CCP 06 (dwarf) and the

Fig. 9.14 Peduncle of the cashew clone CCP 1001 evaluated under non-irrigated conditions at the Paraipaba experimental station (*See* Color Insert)

CP 07 genotype (common type). It is a 3.41 m tall and 7.67 m wide canopy tree by the sixth year (Fig. 9.15). The nut and peeled kernels (including natural humidity) average weight are, respectively, 11.2 g and the 2.9 g. The percentage of kernel/nut is 26.5% and the split kernel in industrial processing is 4.3%. The whole kernel after industrial peeling is 80%. The average yield is 1,261.7 kg/ha of nuts and 5,590 kg/ha of apples in non-irrigated crop, by the 6th year. The yellow apple averages 111 g (Fig. 9.16) and is not appropriated to the fresh fruit market (Barros et al. 2000; Paiva et al. 2003). This clone was released in 1995 for non-irrigated crop.

Fig. 9.15 Plant of the cashew clone EMBRAPA 50 evaluated under non-irrigated conditions at the Pacajus experimental station (*See* Color Insert)

Fig. 9.16 Peduncle of the cashew clone EMBRAPA 50 evaluated under non-irrigated conditions at the Pacajus experimental station (*See* Color Insert)

Embrapa 51

This clone is the result of a phenotypic selection on "polycrossed" progenies of precocious dwarf type, followed by clonal evaluation. The experimental procedure was the same to the clone Embrapa 50. It is a 3.52 m tall and 7.79 m wide canopy tree, by the sixth year (Fig. 9.17). The nut and peeled kernels (including natural humidity) average weight are, respectively, 10.4 g and the 2.6 g. The nut and peeled kernels (including natural humidity) average weight are, respectively, 10.4 g and the 2.6 g. The percentage of kernel/nut is 24.5% and the split kernel in industrial processing is 1.3%. The whole kernel after industrial peeling is 85%. The average yield is 1,255.6 kg/ha of nuts and 8,700 kg/ha of apples in non-irrigated crop by the

Fig. 9.17 Plant of the cashew clone EMBRAPA 51 evaluated under non-irrigated conditions at the Pacajus experimental station (*See* Color Insert)

Fig. 9.18 Peduncle of the cashew clone EMBRAPA 51 evaluated under non-irrigated conditions at the Pacajus experimental station (*See* Color Insert)

sixth year. The red, pear-shaped apple averages 104 g (Fig. 9.18) and, in spite of the favourable colour, is not appropriated to the fresh fruit market, (Barros et al. 2000; Paiva et al. 2003). This clone was released in 1995 for non-irrigated crops.

BRS 189

This clone is the result of a phenotypic selection onto a progeny originated from a crossing between the dwarf clones CCP 76 and the CCP 1001 followed by clonal evaluation. The experimental procedure was the same as the clone Embrapa 50. It is a 3.16 m tall and 5.9 m wide canopy tree by the third year (Fig. 9.19). The nut and peeled kernels (including natural humidity) average weight are, respectively, 7.9 g and the 2.1 g. The average yield is 1,960.2 kg/ha of nuts and 12,738 kg/ha of apples in irrigated crop by the third year. The reddish, pear-shaped apple of an average of 155 g (Fig. 9.20) is adequate to the fresh fruit market if the main traits related

Fig. 9.19 Plant of the cashew clone BRS 189 evaluated under irrigated conditions on the farm of a producer (*See* Color Insert)

Fig. 9.20 Peduncle of the cashew clone BRS 189 evaluated under irrigated conditions on the farm of a producer (*See* Color Insert)

to quality present are: soluble solids – 13.3 Brix; titrable acidity (TTA) – 0.40%; vitamin C – 251.86 mg/100g of pulp; and oligomerous tannin – 0.30%. In general, tannin contents are lower than average in the known cashew nut germplasm. This clone was released specifically for fresh fruit in irrigated crop (Barros et al. 2002).

BRS 226

This clone is a result of an individual phenotypic selection onto progenies followed by clonal evaluation. The progenies was originated from genotypes collected in a survey in a commercial orchard with c.a. 1,110 ha of seed propagated plants in a semi-arid environment. The clonal evaluation was carried at the same place of origin, aimed at identifying clones with some drought tolerance. It is a 1.24 m tall and 2.2 m wide canopy tree by the fourth year (Fig. 9.21). The nut and peeled kernels (including natural humidity) average weight are, respectively, 9.75 g and the 2.7 g. The percentage of kernel/nut is 22.13% and the split kernel in industrial processing is 8.24%. The whole kernel after industrial peeling is 86.7%. The nuts yield by the fourth year is 469 kg/ha in non-irrigated crop (Paiva et al. 2002). The pear-shaped apple colour is orange and it averages 102 g (Fig. 9.22). The content of soluble solids is 13.8°Brix, titrable acidity (TTA) is 0.52%, vitamin C is 356.13 mg/100g of pulp and oligomerous tannin is 0.80%. This clone is recommended for nut purpose in the semi-arid region of Piauí and similar environments.

Tables 9.8 and 9.9 show, respectively, the nuts and apples agro-industrial indicators and the yield evolution of BRS 226 and CCP 76 (control) clones in the experiment carried out at a private orchard in the Pio IX county, Piaui state, a semi-arid environment (Paiva et al. 2001, 2002) (Table 9.10).

Fig. 9.21 Fruit-bearing plant of the cashew clone BRS 226 evaluated under non-irrigated conditions on the farm of a producer (*See* Color Insert)

Fig. 9.22 Peduncle of the cashew clone BRS 226 evaluated under non-irrigated conditions on the farm of a producer (*See* Color Insert)

Table 9.8 Agroindustrial indicators for cashew nuts of BRS 226 and CCP clones in Pio IX county at Piaui state

Characteristics	BRS 226	CCP 76 (control)
Weight of nut (g)	9.75	8.00
Weight of kernel (g)	2.72	2.07
Kernel/nut (%)	22.13	23.98
Whole kernel after peeling (%)	86.69	88.70
Kernel broken after shelling (%)	13.31	11.30
Percentage of split kernel (%)	8.24	10.12

Table 9.9 The cashew apple quality characteristics of BRS 226 and CCP clones in Pio IX county at Piaui state

Characteristics	BRS 226	CCP 76 (control)
Weight of cashew apple (g)	102.6	90.33
Colour	Light orange	Dark orange
Shape	Pear-shaped	Pear-shaped
Total soluble solids – TSS (°brix)	13.8	12.0
Total titrable acidity – TTA (%)	0.52	0.33
TSS/TTA	26.54	36.36
Vitamin C content (mg/100 g of pulp)	356.13	267.81
Oligomerous tannin (%)	0.80	0.58

Table 9.10 Evolution of cashew nut production of BRS 226 and CCP clones in Pio IX county at Piaui state

	Production (kg/ha/year)				
Clone	1st yield	2nd yield	3rd yield	Total (kg)	% over the control
BRS 226	234.6	243.4	469.6	947.6	153.7
CCP 76 (control)	198.5	173.2	245.0	616.7	100

BRS 265

This clone is a result of an individual phenotypic selection onto a progeny originated from the dwarf clone CCP 76, openly pollinated, followed by clonal evaluation in two environments. The main characteristics of this clone are plants 2.5 m tall and 3.6 m of wide canopy tree by the fifth year (Fig. 9.23); nuts and peeled kernels (including natural humidity) average weight, respectively, of 12.5 and 2.56 g; and

Fig. 9.23 Fruit-bearing plant of the cashew clone BRS 265 evaluated under non-irrigated conditions at the Pacajus experimental station (*See* Color Insert)

Fig. 9.24 Peduncle of the cashew clone BRS 265 evaluated under non-irrigated conditions at the Pacajus experimental station (*See* Color Insert)

average yield of 654.0 kg/ha of nuts and 6,200 kg/ha of apples in non-irrigated crop by the third year. The percentage of kernel/nut is 21.3% and the split kernel in industrial processing is 1.2%. The whole kernel after industrial peeling is 98%. The red, pear-shaped apple with an average of 118 g (Fig. 9.24) looks suitable for fresh fruit market. Its quality attributes are: soluble solids – 12.9°Brix; titrable acidity (TTA) – 0.22%; vitamin C – 210.2 mg/100g of pulp; and oligomerous tannin – 0.24%. The tannin content is lower than the average of the available germplasm in Brazil. This clone was released in 2005 for non-irrigated crop.

9.8 Marker-Assisted Selection

In spite of commendable progress attained by conventional breeding towards phenotypic selection, progeny testing, hybridisation and clonal selection the task of cashew breeding has been significant and challenging due to problems like

requirement of large area, longer time consumed to gain achievements and laborious man power requirement. As a quick and dependable methodology, marker-assisted selection (MAS) has been an important instrument to overcome these difficulties. The basic principle of this method relies on those genes for economic traits that are difficult to score on the basis of morphological data.

To date, only few molecular studies have been done in cashew. Random amplified polymorphic DNA (RAPD) has been used to identify considerable differences among four dwarf cashew clones (CCP 06, CCP 09, CCP 76 and CCP 1001) and to determine genetic diversity within and between populations of vivid geographic origin (Neto et al. 1995; Mneney et al. 2001). Polymorphisms were detected among the Tanzanian lines and between and within the geographically diverse lines. Genotypes from India, Mozambique and Tanzania presented the closest relationship, while accessions from Brazil were the most distinctive. Also, Dhanaraj et al. (2002) by using RAPD markers technique, estimated significant diversity among 90 cashew accessions from the National Cashew Gene Bank in India. Archak et al. (2003a,b) applied ISSR (inter simple sequence repeats), AFLP (amplified fragment length polymorphism) and RAPD in commercial cashew varieties of India and identified distinction among all materials while AFLP provided better information in cashew genetic analysis.

The development of genetic mapping is one of the most important applications of molecular markers for plant breeding (Ferreira and Grattapaglia 1996). The provision of genetic maps allows for the identification of marker genes or genetic regions associated with desirable traits and is a key tool in the genetic characterisation of complex characteristics. Ideally, a primary genetic linkage map should contain polymorphic marker loci that are easy to score and regularly distributed all over a genome (Bhattacharya et al. 1999).

The common method used to build a genetic map is through raising an F_1 population by crossing between two inbred lines and subsequently a segregating population (F_2) by self-pollination. DNA is extracted from tissues from these populations (F_1, F_2 and parents) and analysed. However, in cashew, each tree is a natural heterozygotic combination. Therefore, a cross between two individuals produces heterogeneous F_1 population and may behave similarly to an F_2 segregating progeny from a hybrid of two homozygotes (Ferreira and Grattapaglia 1996).

The association of molecular markers with desired phenotype of an agronomic trait is a powerful and effective application of molecular biology to plant breeding (Grattapaglia et al. 1996). Genetic mapping has been used for investigations to identify and locate loci contributing for continuously varying traits known as quantitative trait loci (QTLs) (Hospital 2003).

To begin with, Cavalcanti (2004) using AFLP and microsatellite markers in an F_1 mapping population of 85 individuals originated from a cross of CP 1001 (dwarf commercial clone) and CP 96 (giant genotype) that made two linkage genetic maps. In these maps, the female map (CP 1001) contains 122 markers over 19 linkage groups and the male map (CP 96) comprises 120 markers assembled over 23 linkage groups. The estimated total map distance of the female map is 1050.6 cM, whereas the male map spans 944.7 cM. Moreover, Cavalcanti (2004) identified three QTLs (quantitative trait loci): one for plant height, one for canopy diameter and one

for black mould resistance with an explained phenotypic variation of 22.8, 9.6 and 21.8%, respectively. This author comments that these results provide the groundwork for future marker-assisted selection in cashew and will have particular value in allowing early selection for desired phenotypes leading to accelerate the breeding progress.

9.9 Breeding Perspectives

Success in cashew exploitation in different agro-ecosystems depends on the availability of genotypes adapted to environments. Breeding not only deals with the development of techniques and practices capable of reducing production costs in the commercial orchards, but also increases post-harvest quality of the produce. The new trends demonstrate that irrigation may be useful if there are responsive clones and economic utilisation apple, mainly in the fresh fruit market. So considering the increasing acceptance and good prices of the cashew apple in the fresh market, the cashew business may become more profitable once it aggregates more value to the industry's primary product of exploitation – the nut. Therefore, for some growers, cashew apple will likely be more important than nut. For growers interested preferentially in the apple, the fruit-set must last as long as possible. With the aid of genetic improvement of specific characters inherent to the species it is possible to select clones for irrigated crop with more uniform production of cashew apple. This will also help the producers to aggregate value to their businesses.

Brazil is a continental country with diverse environments. Cashew crop has been introduced into different agro-ecosystems because of its potential for generating employment and income in areas with few economically feasible alternatives. The expansion of cultivated areas has been done utilising basically two clones with a significant risk of genetic vulnerability. The solution is a comprehensive improvement programme for developing clones adaptable to new environments.

In the last three decades, the effort for breeding was centred on dwarf genotype and new genetic gains are still expected since complete variability was not exploited. On the other hand, common type with a number of favourable characteristics towards non-irrigated conditions needs to be included as an integral component in the breeding programmes.

Finally, the use of biotechnology can change the current cashew-breeding panorama by producing new knowledge regarding cashew biology and genetics. This would accelerate the production of new clones and rootstock with desirable quantitative and qualitative attributes.

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Part II
Oil Crops

Chapter 10
Coconut Breeding

Pons Batugal, Roland Bourdeix and Luc Baudouin

10.1 Introduction

The coconut (*Cocos nucifera* L.) is popularly known as a 'tree of life' because of its various economic uses and importance in sustaining the life of the people who grow them. The stem is traditionally used for timber, the leaves for thatch roofing and handicrafts, the coconut sap from spathe processed into vinegar, wine or sugar, the kernel as source of oil and coco cream/milk, the husks as source of fibre/coir for various uses and the coconut water for beverage, vinegar and also wine. The coconut shells, which are traditionally converted into charcoal for the household fuel are now processed into various handicrafts and are usually promoted as tourist souvenir items in Asia-Pacific countries. The coconut palm is very resilient as it can withstand natural calamities like typhoon and flooding. It could survive with minimal care although its yield performance fluctuates depending on the level of inputs and technology applied, particularly fertilisers.

Coconut is planted in about 12 million hectares globally of which 85% are grown in the Asia-Pacific region.

Table 10.1 shows the major coconut growing countries and the area of coconuts grown. Most coconut production (about 96%) is in the hands of smallholders tending 4 hectares or less of land, in many cases not owned by them (share-cropping). Most of the standing coconuts are landraces or local cultivars that were selected by the farmers themselves from existing local populations. Copra (dried kernel from which oil is extracted) yields from these farms range from 0.3 to 1.5 t ha^{-1} (Batugal and Oliver, 2003).

Introduced or exotic cultivars are mostly found in research stations for characterisation and on farm evaluation or for testing as breeding materials. From 1950 to 1993, more than 400 hybrids were developed worldwide under established coconut improvement programmes using various breeding strategies (Bourdeix, 1999). There were also instances where 'accidental' hybrids due to

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Table 10.1 Area of coconut production worldwide, 1999–2003 (in 1,000 Ha)

Country	1999	2000	2001	2002	2003
A. APCC countries	10,320	10,300	10,378	10,431	10,618
F.S. Micronesia	17	17	17	17	17
Fiji	54	54	65	65	60
India	1,755	1,768	1,840	1,892	1,843
Indonesia	3,679	3,696	3,701	3,701	3,883
Kiribati	26	25	25	25	65
Malaysia	171	164	159	159	132
Marshall Island	8	7	7	7	7
Papua New Guinea	260	260	260	260	260
Philippines	3,116	3,119	3,120	3,120	3,214
Samoa	92	96	96	96	96
Solomon Islands	59	59	59	59	59
Sri Lanka	442	442	442	442	422
Thailand	372	325	326	327	328
Vanuatu	96	96	96	96	96
Vietnam	173	172	165	165	136
B. Other countries	1,415	1,411	1,417	1,444	1,441
Africa	670	656	657	650	636
Benin	12	12	12	12	12
Comoros	26	26	26	26	27
Ghana	54	54	55	55	55
Ivory Coast	46	46	46	46	30
Madagascar.	33	33	33	33	33
Mozambique	105	90	90	70	70
Nigeria	36	36	36	49	49
Tanzania	310	310	310	310	310
Others	48	49	49	49	50
America	589	599	601	629	631
Brazil	240	262	263	263	271
Dominican Rep.	37	28	28	37	40
Jamaica	35	35	35	51	51
Mexico	173	170	171	171	148
Venezuela	18	18	18	21	21
Others	86	86	86	86	100
Asia	96	98	101	105	120
Bangladesh	32	32	32	31	31
China	20	20	20	25	27
Myanmar	32	34	37	37	41
Others	12	12	12	12	21
Pacific	60	58	58	60	54
Fr. Polynesia	20	18	18	20	20
Others	40	40	40	40	34
Total	11,735	11,711	11,795	11,875	12,059

Source: APCC 2003 Coconut Statistical Yearbook.

natural out-crossing were identified and selected by the farmers. In the 1990s, with the availability of characterised germplasm from genebanks, hybridisation towards developing improved varieties intensified. Research stations reported copra yields of 2–6 t ha^{-1} from their hybrids. However, hybrids are seldom grown commercially

due to the lack of information on their availability, lack of planting materials and high input requirements. Hence, comparing the national yield average in farmers' fields and those of research stations in 15 coconut growing countries, the technology gap in terms of nuts or copra yield was estimated at 33–84% (Batugal and Oliver, 2003).

Many countries with national breeding programmes tend to develop hybrids from 'proven' single crosses based on results known from advanced breeding programmes to eliminate the need for combining ability tests of potential progenitors, which would require at least 12 years. However, the coconut is highly polymorphic, i.e. its performance is determined not only by its genes but also by environmental factors. Hence, results of progeny testing from same parental population vary within and across countries.

This chapter presents the current strategies and developments in coconut breeding, and the supporting strategies and technologies that are being applied to facilitate the breeding efforts of the coconut producing countries. It also describes the efforts being done in collecting the various ecotypes to conserve the coconut genetic diversity and facilitate its use for developing the desired ideotype which, for most breeders, would mean varieties with broad adaptability, pests and disease resistance and high yield.

10.2 Origin and Botany

10.2.1 Origin and Dissemination

The coconut belongs to Family Palmae of the monocotyledons under the sub-family *cocoidae* that includes 27 genera and 600 species. It is a diploid with 32 chromosomes ($2n = 32$) and the sole species of the genus Cocos. It is a tropical palm; traditionally seed propagated and is slow growing (with reproductive maturity at 3–10 years depending on the genotype and environment). Being monotypic, hybrid cultivars are generally a product of intra-specific crosses.

The coconut has no wild form and early botanists believed that its origin was from America while others considered the Polynesian and India-Pacific Islands as its ancestral home (Child, 1974). However, these early speculations have been later abandoned in favour of South and Southeast Asian origin based on fossil nut deposits, recorded early concentration of cultivation, old literatures citing ethnocultural ceremonies involving the coconuts, etc. Nevertheless, its precise original home within this region is still unknown. Menon and Pandalai (1960) cited that the coconut appeared to have moved eastwards from its southeast ancestral region towards the Pacific and further into America. Towards the west, it moved to India and Madagascar.

Evidences suggests that coconuts spread by sea currents from its coastal centre to many island groups. Even at the present age, nuts could be seen sometimes floating or washed ashore in coastal areas. In addition, voyagers used to carry coconuts as

source of food and drink that enhanced the introduction of the crop to their land destinations. Nowadays, distribution is through direct germplasm exchange among coconut growing countries or importation from genebanks.

Recent studies showed that coconut populations could be differentiated in two main geographical groups based on molecular markers: (1) The Pacific group, which covers a broad area, ranging from Southeast Asia to the South Pacific and also the West coast of America; (2) the Indo-Atlantic group that includes India, Africa, the Caribbean islands and the Atlantic coast of America (see section on molecular techniques).

10.2.2 Morphological Description

Coconuts are generally classified as Tall or Dwarf (Santos et al. 1996). The Talls, sometimes referred to as var. *typica* (Nar.), are widely distributed, can grow more than 50 cm annually, flower at 6–10 years with an economic life of 60–70 years. Dwarfs, sometimes referred to as var. *nana* (Griff.) are believed to be mutants from Tall types with shorter stature (about 8–10 m when 20 years old. See Fig. 10.1). They have a shorter productive life of 30–40 years but usually start flowering in the third year at less than one meter high. Occasionally, natural crosses that occurred sporadically in traditional populations between the Talls and the Dwarfs produced 'intermediate' types that are phenotypically distinct from either the Talls or the Dwarfs. These open-pollinated hybrids may become fixed as 'semi-Talls', which have the same mating behavior as Dwarfs but grow faster.

The coconut grows from a single shoot meristem or terminal bud (commonly called the 'cabbage' in all the Palmae) at the apex of the plant. The stem is erect, unbranched and cylindrical with no outer cambium layer but nevertheless unusually sturdy and can tolerate adverse conditions like strong wind, typhoon and even ring barking because of its numerous vascular bundles. It is anchored by numerous adventitious roots that are produced from the 'swollen' basal (known as 'bole') part of the stem. The top of the trunk is surmounted by a radiating compact crown (which could either be spherical, semi-spherical, drooping or erect) of large, thick cuticled parappinate leaves. The petiole or leaf stalk is directly attached to the stem that naturally falls when it reaches the tail end of its mature phase leaving leaf scars on the trunk. A rough estimate of the age can be made by counting the leaf scars on a coconut trunk. A coconut palm may produce 14 leaves a year but in old age an average of 12 is probably reasonable as a basis for estimating age, but this may also vary depending on the genotype (Child, 1974).

The Dwarfs may start to flower as early as three years while Talls flower in five years under favourable conditions. A flower cluster or inflorescence enclosed in a sheath (called 'spadix') is produced in each leaf axil in continuous succession. Each inflorescence consists of 20–30 branches (rachillae) but some may abort due to unfavourable climatic conditions and/or poor cultural management. The coconut palm is monoecious, that is, its inflorescence carries both staminate (male) and

Fig. 10.1A The Vanuatu Tall (*See* Color Insert)

pistillate (female) flowers (Frankel and Galun, 1977). The female flowers (also known as 'button nuts') are situated at the base of each rachilla while most of the male flowers could be found on the apical portion of the inflorescence and beside the pistillate flowers. After fertilisation, the button nuts develop into fruits, forming a bunch and mature in about 12 months into a nearly round or spherical-shaped nuts. The ripe nut is a large drupe with the solid white kernel (endosperm) encased with a hard shell (endocarp). At the basal end of the fruit, there are three 'eyes' one of which is soft where the plumule or the shoot appears during germination. A coat of fibrous mass (mesocarp), that is, the husk from which coir is obtained, further protects the shell. For young or green coconut, the mesocarp is white and firm and the kernel is thin and jelly-like. Water or liquid endosperm is found inside the cavity of the kernel, which decreases towards the end of maturation.

The green nuts are either harvested for its water or for its soft endosperm for household or commercial uses or left to further mature for processing into copra

Fig. 10.1B Tahiti Red Dwarf; Pictures by R. Bourdeix (*See* Color Insert)

and for other purposes. Some of the mature nuts are reserved for use as seed-nuts. Seed storage is not commonly practiced because of the recalcitrant nature and the large size of the nuts.

10.2.3 Anthesis and Pollination

Generally Talls, being protandrous, shed pollen prior to stigma receptivity. They are generally considered as allogamous. Nevertheless, selfing is possible through inter-spadix pollination because of the overlapping between the female phase of an inflorescence and the male phase of the next inflorescence. The speed of emission of inflorescences varies according to genotype and environment with a great seasonal variation; so does the selfing rate. On the other hand, the Dwarfs

are generally considered autogamous/homogamous as stamens and pistils mature simultaneously. Thus, Dwarfs can shed and receive pollen at the same time resulting in inbreeding. Apart from their short stem, most of the Dwarfs show a combination of common characteristics: preference to autogamy, sensitivity to environmental stresses, small-sized organs, precocity and rapid emission of inflorescence. Because of the last three characteristics, the Dwarfs play an important role in hybridisation programmes. However, the genetic determinant of coconut dwarfism is still unknown.

The bisexual nature of the Talls and the Dwarfs allow manipulation of pollination to secure the desired level of genetic introgression with the Talls as source of heterozygous genotypes while the Dwarfs provide the progenitors of homozygosity.

10.3 Genetic Resources Utilisation

Although coconut is monotypic, considerable diversity can be observed in the existing populations. The varieties can be differentiated qualitatively (by stature, i.e. whether tall or dwarf; leaf configuration (formation); size, shapes and colour of the nuts (Fig. 10.2); and pests and disease resistance) and quantitatively (i.e. precocity of flowering, vertical growth of the stem, fruit setting or number of bunches, number of nuts, weight and composition of the fruit). Most collecting expeditions and conservation centres use these parameters in characterising their coconut germplasm. However, the genetic bases of these phenotypic variations, whether it's simple or complex, are still generally unknown in coconut. Nevertheless, initial molecular studies have shown that polymorphisms in DNA sequences are present and are currently being used for genetic fingerprinting of conserved germplasm to avoid duplications of accessions in genebanks (see section on molecular techniques). It is also important in determining cases of environmental covariance where populations, which are genetically different, resemble one another due to shared environment.

In 1992, the International Plant Genetic Resources Institute (IPGRI) organized the International Coconut Genetic Resources Network (COGENT), a global network of 38 coconut growing countries (Batugal et al., 2005). The latter has coordinated the collection of important coconut varieties worldwide and their conservation in national coconut field genebanks with important duplicates maintained at the COGENT's multi-site International Coconut Genebank (ICG). The ICG is a series of regional genebanks hosted and maintained by India for South Asia; Indonesia for Southeast and East Asia; Papua New Guinea for the South Pacific; Côte d'Ivoire for Africa and the Indian Ocean; and Brazil for Latin America and the Caribbean (Ramanatha Rao and Batugal, 1998). Based on COGENT's International Coconut Genetic Resources Database (CGRD), the total number of germplasm collected and conserved increased from 761 in 1994 to 1,416 accessions in 2003.

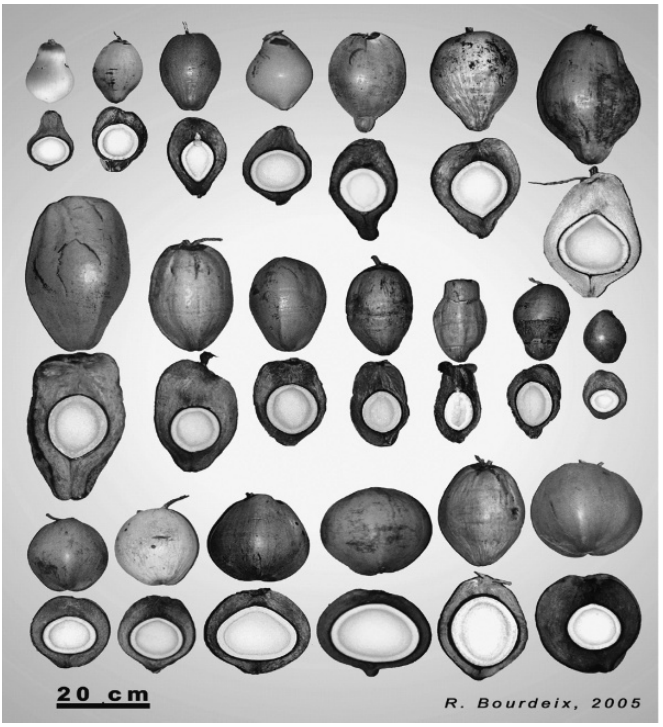

Fig. 10.2 Variability of the shapes of coconut fruits from different varieties (Picture by R.Boudeix) (*See* Color Insert)

From left to right, then top to bottom:

First rank:
- Papua Yellow Dwarf, Papua New Guinea
- Tahiti Red dwarf, French Polynesia
- Madang Brown Dwarf, Papua New Guinea
- Cameroon Red Dwarf, Cameroon
- Spicata Tall Samoa, Western Samoa
- Rotuman Tall, Fiji
- Rennell Tall, Solomon Islands

Second rank:
- Niu Afa Tall, Western Samoa
- Comoro Moheli Tall, Comoro Island
- Sri Lanka Tall Ambakelle, Sri Lanka
- West African Tall Akabo, Côte d'Ivoire
- Tuvalu Tall Fuafatu, Tuvalu Island
- West African Tall Mensah, Côte d'Ivoire
- Miccro Laccadives Tall, India

Third rank:
- Vanuatu Tall, Vanuatu
- Malayan Yellow Dwarf, Malaysia
- Malayan Tall, Malaysia
- Tagnanan Tall, the Philippines
- Tampakan Tall, the Philippines
- Kappadam Tall, India

Table 10.2 Summary information of conserved germplasm in the COGENT International Coconut Genetic Resources Database* (Source: Hamelin, 2003)

Site	Number of accessions	$25 < P \leq 75$	$25 < E \leq 75$	With pictures	With molecular data
1. CNRA Marc Delorme Research Station, Port-Bouët, Côte d'Ivoire	99	92	71	73	67
2. Coconut Programme, OPRI, Sekondi, Ghana	16		4	15	14
3. CRC, Sémé Podji, Benin	4	4	4	4	3
4. National Coconut Development Programme, Dar es Salaam, Tanzania	72	71	69	35	33
AFRICAN REGION	191	103	148	127	117
5. Centro de Investigacion Cientifica de Yucatan, Merida, Mexico	20	20	1	1	2
6. Coconut Industry Board, Kingston, Jamaica	60	16	58	32	36
7. EMBRAPA, Aracaju and Betume Stations, Brazil	16	16	16	10	10
LATIN AMERICA-CARIBBEAN REGION	96	52	75	43	48
8. BARI, Gazipur, Bangladesh	40	18	37		
9. Coconut Research Institute, Lunuwilla, Sri Lanka	78	78	64	5	10
10. CPCRI, Kasaragod, India	212	141	211	76	52
Res. Station, Islamabad, Pakistan	32				
SOUTH ASIAN REGION	362	237	312	81	62
11. Cocoa and Coconut Institute, Rabaul, Papua New Guinea	3		3	5	30
12. Stewart Research Station, Madang, Papua New Guinea	54	31	54	3	2
13. Ministry of Agriculture, Nuku'alofa, Tonga	7		1	2	2
14. Saraoutou Research Station, Santo, Vanuatu	79	71	11	48	53
15. Taveuni Coconut Centre, Taveuni, Fiji	11	8	7	5	5
16. Res. Station, Apia, Western Samoa	9		9	4	3
17. Res. Station, Yandina, Solomon Islands	21	4	21	10	11
SOUTH PACIFIC REGION	184	114	106	77	106
18. Res. Station, Wenchang, China	17	15	17		14
19. Department of Agriculture, Sabah, Malaysia	45	23	30	23	19
20. MARDI, Hilir Perak, Perak, Malaysia	44	34	39	40	38
21. Bone Bone Experimental Garden, Manado, S. Sulawesi, Indonesia	41	35	41		
22. Mapanget Experimental Garden, Manado, N. Sulawesi, Indonesia	74	74	45	14	17

Table 10.2 (continued)

Site	Number of accessions	$25 < P \leq 75$	$25 < E \leq 75$	With pictures	With molecular data
23. Pakuwon Experimental Garden, Bogor, W. Java, Indonesia	25	22	25	8	10
24. Sikijang Experimental Garden, Pekanbaru, Indonesia	30	30	30	3	5
25. Philippine Coconut Authority, Zamboanga, The Philippines	224	221	219	194	51
26. Chumphon Horticultural 27. Research Centre, Chumphon, Thailand	52	42	52	9	8
28. Dong Go Experimental Centre, Ben Tre Province, Vietnam	31	30	16	9	8
SOUTHEAST ASIAN REGION	583	526	514	300	170
TOTAL FOR ALL REGIONS	1, 416	1, 032	1, 155	628	503

Number of accessions per site or per region according to the percentage of passport descriptors (P) and evaluation descriptors (E) filled in and the number of pictures and molecular data.

(Table 10.2). These are conserved in 28 government genebanks or conservation sites in 22 countries worldwide. Most of the conserved germplasm have been partially characterised, i.e. 82% with morphological descriptions and 36% with molecular data. As of 2003, the CGRD listed 599 Talls, 111 Dwarfs and 1 semi-Tall cultivar and a few cross-fertilising Dwarfs (Hamelin, 2003). However, less than 5% (< 60) of the listed germplasm are actually being used as parents in national breeding programmes of the COGENT member countries (Batugal, 2004). Since most of the differentiation among the accessions is based on morphological data, the genetic structure of the conserved germplasm is yet to be fully understood that limits the exploitation of their diversity for breeding purposes.

Some of the more popular hybrids have been previously tested internationally under various ecological conditions and results showed that there is no 'universal' hybrid because of low plasticity in crop response. Nonetheless, hybrids generally perform better than traditional varieties under good rainfall and soil conditions. The more popular parental accessions for hybridisation were the West African Tall (WAT), Rennell Island Tall (RIT), Vanuatu Tall (VTT), Malayan Yellow Dwarf (MYD), Malayan Red Dwarf (MRD), Malayan Green Dwarf (MGD) and Cameroon Red Dwarf (CRD). With the published characterisation data (quantitative and qualitative descriptions of major traits) on most of the catalogued accessions of the CGRD and the availability of conserved germplasm in ICG and national genebanks, more accessions are currently being incorporated in national working collections for germplasm improvement and utilisation purposes. These developments have given new options to breeders to incorporate more accessions into their breeding programmes.

A bibliographical study was recently conducted on some rare types of coconut palms known as 'sweet husk' or SWH; in their case, the husk of unripe fruits, which is usually tough and astringent, is tender, edible and sweet. A sufficiently ripe fruit can be husked by hand, which is impossible with an ordinary coconut. The only place these rare types are found in any appreciable quantities is the islet of Onoiki in the Ha'apai group of Tonga. The islet is so small that it does not figure on most of the maps. In 1848, it was reported that coconuts having a circumference of more than 60 cm have been found on another islet of Niu-foi in Tonga (Teuira Henry, 1928). This islet has even taken the name of the newly discovered coconut variety as its name 'Niu-foi' means 'new coconut'. Planting on islets seems to have played some role in varietal creation and conservation. The geographical remoteness of the islets has been a reproductive barrier that has enabled new coconut varieties to become fixed.

10.4 Current Status of Breeding Efforts

10.4.1 Breeding Objectives

There are three major constraints to sustainable world coconut production namely, (1) lack of adapted, high yielding varieties; (2) low level of yield stability; and (3) low farmer productivity (Batugal, 1999). Hence, the major coconut growing

countries focus on breeding superior varieties with high yield, high oil content, disease resistance and tolerance to biotic stresses. Yields are generally measured in terms of the number of nuts and copra/nut. Other yield parameters may include nut size, weight of whole nut, dehusked nut, shell and water content. The oil content is derived by drying the kernel, extracting the oil using oil expellers and determining the percentage of oil over dry matter.

Breeding for disease resistance is given more emphasis in African and South American countries because of the presence of the phytoplasma-caused lethal yellowing disease in these regions. Vanuatu is similarly developing varieties with resistance against coconut foliar decay. Some countries like India, Sri Lanka and Tanzania pay attention to drought tolerance since coconuts are generally grown in marginal, rain-fed areas. On the other hand, The Wenchang Coconut Research Institute in Hainan (China) is developing cold resistant varieties for its low-temperature (about 10°C during the winter) growing areas in Southern China. The Caribbean and Pacific countries are interested in developing cyclone tolerant varieties because their open positions make them wind disaster-prone areas.

10.4.2 Breeding Programmes

In 1996, the first international gathering of coconut breeders was held in Côte d'Ivoire to standardise the research techniques on coconut breeding and present the coconut breeding programmes being implemented in their home countries (Batugal and Ramanatha Rao, 1998). Most of the national breeding programmes were focused on evaluating local cultivars versus introduced varieties imported from the more advanced breeding centres like the Marc Delorme research station in Côte d'Ivoire. However, a follow up survey conducted by COGENT in 2001–2003 (Batugal, 2004a) indicated that locally produced hybrids became more prominent in national varietal performance trials. In addition to progeny testing many countries conducted: (1) morphological and genotypic characterisation of breeding accessions; (2) testing of introduced/collected breeding materials for general and specific combining abilities; (3) selection/acquisition of mother palms and sources of pollen; (4) pollination of selected mother palms to produce the desired crosses; and (5) production of hybrid nuts for field testing.

The performance of coconut hybrids in selected countries of Asia-Pacific, Africa and Latin America are summarised below.

China. The Wenchang Coconut Research Institute's sole recommended hybrid is a cross between Malayan Yellow Dwarf (MYD) and the local Hainan Tall (HAT) variety. This MYD × HAT hybrid (WY78F1) exhibited early flowering (3–4 years) and 3–4 fold increase in terms of harvested nuts (80/palm/year) and copra ($4\,t\,ha^{-1}\,yr^{-1}$).

Philippines. The Philippine Coconut Authority (PCA) recommended nine hybrids derived from single crosses involving the local cultivars Catigan Green Dwarf (CAT), Tagnanan Tall (TAGT), Baybay Tall (BAYT), Laguna Tall (LAGT),

Bago-Oshiro Tall (BAOT) and the introduced varieties Malayan Red Dwarf (MRD) and Polynesian Tall (PYT). Most of these recommended hybrids started flowering from the third to fourth year onwards. The average number of nuts per palm ranged from 117 to 155 and copra yield per hectare, from 4 to 6 tons. The local Tall BAYT was comparatively good, producing 114 nuts per palm with a copra yield of 5 t ha^{-1}. Among the nine hybrids, MRD × TAGT (PCA 15–2) and MRD × BAYT (PCA15-3) were outstanding giving the highest number of nuts (144–155/palm) and copra yield (6 t ha^{-1}).

Thailand. The Chumphon Horticulture Research Centre (CHRC) of the Horticulture Research Institute recommends three high yielding hybrids: Sawi Hybrid No.1 (an introduced hybrid known as PB 121 or MAWA), the locally developed hybrids Chumphon Hybrid No.60 (Maphrao Yai or Thai Tall × West African Tall) and Chumphon Hybrid No. 2 (MYD × Thai Tall). A trial comparing the locally developed hybrids with the local Thai Tall (THT) in 1975 showed that THT yielded the least. Nut and copra yields of the recommended hybrids ranged from 80 to 126/palm and from 3.4 to 4.2 t ha^{-1}, respectively.

Vietnam. The Vietnam Oil Plant Institute (OPI) is recommending seven introduced high yielding hybrids in the country which have significantly out-yielded the local Tall (Ta). The introduced hybrids were PB111, PB121, PB 132, PB 141, JVA 1, JVA2 and CRIC 65 with nut production ranging from 48 to 69/palm in 1996. The local variety Ta yielded 31–35 nuts in the same year. OPI is currently testing six local hybrids in Dong Go Experimental Centre (Eo × Ta; Tam Quan × Ta; Tam Quan × BAOT) and in Binh Thanh Experimental Station (MYD × Renell Tall; MYD × Palu Tall; MYD × Ta).

Bangladesh. The Agricultural Research Institute (BARI) has developed two high-yielding coconut hybrids: BARI Narikel-1 and BARI Narikel-2. These hybrids are broadly adapted and capable of producing 65–70 nuts/palm throughout Bangladesh. In addition, BARI is recommending two introduced varieties to the country's coconut growing communities, namely: Sri Lanka Tall and Malaysian Yellow Dwarf.

India. The Central Plantation Crops Research Institute (CPCRI) has released the largest number (12) of single-cross hybrids among all the coconut-growing countries involving Chowgat Orange Dwarf (COD), West Coast Tall (WCT), Laccadive Ordinary (LCT), Gangabondam (GBGD), MYD, SS Apricot by KAU (SSAT) and East Coast Tall (ECT). All the hybrids performed better than the traditional cultivar WCT. The recommended hybrids have reported average nut yields of 98–156/palm while the WCT has recorded only 80 nuts per palm. COD × WCT (Chandra Sankara), WCT × SSAT (Kera Sowbagya) and WCT × MYD (Kera Sree) produced the highest copra yields, i.e. more than 4 t ha^{-1} per yr^{-1}.

Sri Lanka. The Coconut Research Institute (CRI-SL) has developed two hybrids, (Sri Lanka Green Dwarf [SLGD] × Sri Lanka Tall [SLT]) SLT × SR and a first generation inbred (SLT × SLT) for its national replanting programme. Their yields ranged from 80 to 125 nuts/palm and from 3.6 to 4.0 t copra/ha. Hybrids' nut yields are double that of the usual yield of the local cultivar, SLT, but their copra content/yields are similar.

Vanuatu. The Vanuatu Research and Training Centre has produced hybrids involving the local cultivars Vanuatu Tall (VTT) and Vanuatu Red Dwarf (VRD), and the introduced varieties Renell Island Tall (RIT) and Brazilian Green Dwarf (BGD). The Malayan Red Dwarf (MRD) was also used as a mother palm for crossing with RIT but the resulting hybrids only performed slightly better (in terms of copra yield) compared to the local VTT and were very susceptible to coconut foliar decay (CFD). The BGD crossed with either RIT or VTT produced the best copra yields of 4.4–5.2 t/ha but they were also found to be very susceptible to CFD. The VRD × VTT hybrids had lower copra yields (3.3–3.7 t ha^{-1}) but were found to be more tolerant against CFD. Both the traditional and improved VTT types had the lowest reported copra yields of 2.6–2.8 t ha^{-1}, but comparable with the hybrid MRD × RIT.

Côte d'Ivoire. The Centre National de Recherche Agronomique (CNRA) Marc Delorme Coconut Station initially identified six outstanding hybrids: PB 213 (WAT × RIT), PB 214 (WAT × VTT), PB121 (MYD × WAT), PB 132 (MRD × TAT or Tahitian Tall), PB123 (MYD × RIT) and PB111 (CRD or Cameroon Red Dwarf × WAT). These hybrids flower very early (40–57 months after field planting) under Côte d'Ivoire conditions. They produced from 100 to 132 nuts/palm/year which is 34%–138% higher compared with the control, i.e. the WAT. Furthermore, their copra yields ranged from 3.15 to 4.8 t ha^{-1} or from 86 to 135% more compared with WAT.

Ghana. All coconut cultivars in Ghana are considered to be at risk from the Cape St. Paul Wilt disease (CSPWD), a lethal yellowing type of disease. Hence, the coconut breeding programme in the country is geared towards developing hybrids resistant or highly tolerant to CSPWD. There are six cultivars and 21 hybrids being tested in four locations: Cape Three Points, Discove, Agona Junction and Akwidae. These varietal resistance trials are still under observation although some of the test materials were already totally infected by the CSPWD.

Tanzania. The Mikocheni Agricultural Research Institute (MARI) is currently testing six hybrids with the local East African Tall (EAT) as the sole pollinator. Mother palms involved Malayan Green Dwarf (MGD), CRD, Pemba Red Dwarf (PRD), MYD, MRD and improved EAT populations. In addition to determining their yield performance, the F_1 progenies are also being evaluated for their resistance to lethal disease and tolerance to drought stress.

Mexico. Coconut research at the Instituto Nacional de Investigacion Agropecuaria Y Forestal is focused on developing hybrids resistant to lethal yellowing disease. Initial hybrids were mainly derived from crosses between MYD and improved Pacific Tall populations. Intra-population crosses of selected Pacific Tall were also done and these are currently being tested.

10.4.3 Constraints to Coconut Breeding

In a perennial plant such as the coconut palm, the constraints connected with its biology increase the cost of the scientific progress and aggravate the consequences of possible errors. In fact, a genetic experiment frequently covers an area of

8 hectares for a minimum period of 12 years. A coconut breeder often analyses the trials established by his/her predecessor and establishes trials for his/her successor. Consequently, coconut research not only needs high financial and human resource investments but also a secure environment. In various countries, many years of research have been lost as a result of different types of calamities and constraints such as fires, floods, revolutions, turnover of personnel or simply the lack of funds leading to termination of the breeding programme. In some cases, due to the very long period between the start and completion of a breeding project, the data from initial years of bearing have been lost even before the completion of the experiment.

Therefore, it is imperative to make sure that the collected data at the research stations will be available and safely kept for many years. These data should be duplicated systematically in two geographically different locations. These may be two different national institutes or a national institute cooperating with a specialised international research institute.

Technically, the main constraint in breeding coconuts, especially the Dwarfs, is the need for emasculation and artificial pollination to produce the desired recombinants. The out-breeding Talls could be grown in isolation such that only the desired parents would have the chance to inter-cross but this will require establishing seed gardens for the desired populations with sufficient spatial barriers. The difficulty and substantial costs of conducting artificial pollination and in maintaining isolated seed gardens justify the need to explore the existence of male-sterility or self-incompatibility in the existing populations and use them to facilitate the production of hybrids. However, to date coconut palms with any of the traits conferring male sterility have not been found.

10.5 Breeding Strategies and Methodologies

10.5.1 Coconut Productivity

In comparing the national yield average in research stations with those in farmers' fields in 15 coconut producing countries, it is estimated that coconut technology gap in terms of nuts and copra yield ranged from 33 to 84%. The significant improvement in productivity in research stations could be attributed to the use of hybrids/improved varieties in conjunction with proper management and cultural practices. The low productivity in farmers' fields could be due to poor cultural management/lack of production inputs and the use of poor quality planting materials. Although hybrids generally performed better than the traditional varieties, they are currently grown in limited areas, less than 0.1 (or even nil) to 14% of cultivated coconut farms in various countries. The poor adoption of coconut hybrids may be attributed to inadequate information dissemination on the availability of improved hybrids/varieties and lack and affordability of planting materials. The above factors that affect coconut productivity should be considered in developing and implementing breeding strategies and technologies.

Table 10.3 Coconut productivity in farmers' field and research stations and area planted to hybrids

Country	Yield/Year (A) Farmers' fields/national average Nuts Copra t/ha	(B) Research station/hybrids Nuts Copra t/ha	Technology gap 100- (A/Bx100)	Area grown to hybrids(% production area)
South Asia				
Bangladesh	21/palm	69/palm	70	Nil
India	6,892/ha	23,700/ha	71	14
Sri Lanka	42/palm	63/palm	33	11
Southeast Asia				
Indonesia	1.1	3.5	69	5
Malaysia	10,000/ha	23,000/ha	57	n.a.
Philippines	0.78	4–6	84	n.a.
Thailand	1.2–1.5	3.0	55	10
Vietnam	38–48/palm	55–80/palm	42	<0.1
South Pacific				
Fiji	0.3–0.5	2.0	80	<5
PNG	0.66	2.8–3.6	80	1.5
China	1.27	3.6	65	1.5
Africa				
Ghana	20/palm	n.a.		3
Tanzania	40/palm	80/palm	50	n.a.
LAC				
Jamaica	0.8	3.7	78	n.a.
Mexico	0.65	4.0	84	1

(Source: P Batugal and R. Bourdeix, 2005).(see Table 10.3, Batugal and deix, 2005)

10.5.2 Breeding for Yield Improvement

The breeding strategies that have been applied to the yield improvement of the coconut palm can be divided into two main sections: intra-varietal breeding strategies (mostly based on the mass selection methods) and hybridisation between varieties (exploitation of hybrid vigour). These two sets of methods and the possible future of coconut breeding programmes are discussed below.

10.5.2.1 Intra-Varietal Breeding Strategies

Mass selection: The majority of world coconut is derived from mass selection informally done by all growers. At the end of the nineteenth century, large plantations were established by importation of fruits from a region known for its production (Ziller, 1962). In most cases, the seed-nuts were selected according to their specific characteristics: some preferred large and heavy fruits (Zuniga et al., 1969), others medium-sized fruits preferably round-shaped (Apacible, 1968). The genetic structure of the coconut populations has been modified by successive selections based on fruit characteristics.

From the breeder's point of view, there are three variants of mass selection based on the reproductive system used – mass selection using open-pollination, selfing or inter-crossing (Bourdeix, 1988a). Mass selection using open-pollination has been practiced the most. The advantage of the method is its simplicity; the seed-nuts are collected from the palms that present attractive characteristics at a certain time or over a period. The progenies resulting from open-pollination are the basis of an improved population that will then undergo other selection cycles. This method leads to variable results. Even in the most favourable cases (yield improvement of 14% per cycle), the drastic selection necessary to obtain an improvement considerably reduces seed-nut production potential. One generation of multiplication is inevitable. Thus, it is better to use this generation for the evaluation of the parents based on the performance of their progeny. The only advantage of mass selection using open-pollination is its simplicity.

Mass selection using inter-crossing appears more effective, as it allows for a strict selection of pollinators while retaining the potential for large seed-nut production. However, there is no experimental result that makes it possible to assess the genetic progress that could be realized from this controlled breeding scheme.

The study on efficiency of mass selection method using open-pollination is characterised by a number of divergent results (from 0 to 14 % improvement per cycle). From 1960 to 1990, this point was the main scientific controversy in the coconut research community. The difference may find its origin in the reproductive character of the Tall varieties. Although the latter are preferentially allogamous, there is the possibility of natural selfing. The rate of selfing increases with the rhythm of inflorescence emission and bunch production. This rhythm depends on the individual vigour of the palm and on climatic conditions. When selecting good-performing palms in the best plots, one may select palms with a higher tendency for selfing. Consequently, their progeny suffers from an inbreeding depression resulting in lower productivity. The rhythm of inflorescence emission also varies with seasons and so do the selfing rate.

In Sri Lanka, a national replanting programme has been in operation in the country for the last four decades. Most of the planting materials were produced from a pool of about 50,000 good mother palms selected from the farmers' fields and experimental stations (Peries, 1998). In such a programme, investigations about the selfing rate are strongly required. If the best palms have a higher selfing rate, a single generation of selfing usually reduces the yield of fruits from 20 to 30%. In Tall types, selfing generally induces an average yield decline of 15–30% without appreciably increasing production homogeneity. There are practical solutions to restrict selfing during the open-pollination process. The first option could be as simple as avoiding the harvesting of seed-nuts during the 3–6 months period within the year where the emission of inflorescences is the fastest. The second alternative is to remove one of the inflorescence within two successive inflorescences to minimise overlaps of pollen dehiscence and stigmatic receptivity.

The level of selection pressure at the nursery stage is also very important. Seedlings derived from self-pollination are generally less vigorous than hybrid

seedlings. It is probable that the selection traditionally conducted by farmers in their fields have the effect of eliminating a significant proportion of seed-nuts resulting from self pollination, although this was never formally demonstrated. The worst situation could be when all the germinated seed-nuts are released to farmers because the demand for planting materials is greater than the capacity of production. Such a situation happens often in development projects where the desire for success can lead to overloaded nurseries.

Within population selection based on progeny test: As in the case of mass selection, the methods described hereunder have a common denominator (i.e. the selected population initially was limited to only one local Tall coconut variety).

The 'prepotency' concept was introduced to indicate coconut palms in which the open pollinated progenies show superiority. Harland (1957) proposed to collect seed-nuts from the prepotent palms. This method corresponds to the starting of a recurrent selection on half-sib families. In 1967, Liyanage published a study dealing with the analysis of the open pollinated progenies of 104 Sri Lanka Tall. The low number of each progeny (nine half-sibs) seems insufficient to safeguard against divergence due to sampling and environmental effects. However, this study made it possible to estimate the effect of a selection based on the open pollinated progenies. The 6 best families among the 104 exhibited 32% higher yield level than the average. This genetic gain, although it might have been overestimated, could be compared to the progress obtained by mass selection. The selection of the 6 highest yielding parents could generate a 14% yield increase of their progeny compared to the average of progenies from all parent palms. This study underlines another limit of mass selection (i.e. progeny tests are indispensable in evaluating the effectiveness of this method). Once these tests have been conducted, it is much better to select the parents on the basis of the progeny value than on the basis of their phenotypic value. The selection of half-sib families (HS) is therefore more effective than the mass selection method.

Harland (1957) also proposed to evaluate and select full-sib families (set apart progenies from only two parent palms). This method exploits within-variety heterosis effect, a phenomenon clearly illustrated by the existence of inbreeding depression. The best full-sib progenies afterwards could be reproduced only by hand-pollination. The problem is that this hand-pollination technique requires bagging the inflorescence and cannot be used economically for mass seed-nuts production. It produces only 40–50 expensive plants per pair of palm per year. This explains why this method has never been developed for direct use. An improvement of this method would be to self each parent palm immediately after the establishment of the trial. Thus, once the interesting pairs have been identified, the hybrids can be reproduced by inter-crossing these selfs using the assisted pollination (Nuce de Lamothe and Rognon, 1972a). The seed-nut production capacity, even if improved, remains insufficient and costly, at least in the first generation. However, it must be noted that the selfs are less productive due to inbreeding depression. Moreover, nut production requires large areas due to the isolation that is needed between seed gardens.

In Indonesia, a programme was undertaken with 43 half-sib families of Mapanget Tall produced by Tammes in 1927 (Tammes, 1955). In this experiment, parents were selected and crossed, sometimes within and other times between families. About 50 progenies were planted from 1957 to 1959 with an initial number of 50 palms each. Extremely promising yields in the order of 40 kg of copra per palm per year were obtained. When the measurements were made in 1975–1979, 59% of the trees had died, clearly favouring the yield of the remaining palms. Converted to full density, the best progeny tested yielded 3.05 tonnes of copra per hectare. However, this trial made it possible to identify four elite families, which have been used for the production of open pollinated seed-nuts.

In Vanuatu, beginning with two Vanuatu Tall populations collected near the Saraoutou research centre, four mass selection cycles by open-pollination or intercrossing were conducted from 1962 to 2002. This programme resulted in improved Vanuatu Tall population intended for distribution to farmers. The main criterion of selection was copra weight per fruit. Because of the strong negative correlation between the number and weight of fruit (Bourdeix, 1988), there was no increase in the number of fruit per palm over the different generations; rather a slight decrease was observed.

All the classical methods based on within-population breeding can be applied to the coconut palm, although the long generation period and the low multiplication rate are strong limiting factors especially for an economically viable seed-nuts production system.

A pragmatic coconut breeder could ask the following question: 'Why focus on a within variety breeding method when the hybrid vigour between varieties gives an opportunity of immediate and appreciable genetic progress?' Nevertheless, the within-population breeding strategy is fully justified. Sometimes, such as in the Vanuatu archipelago, only one local variety is resistant to a lethal disease. In many countries, there is a cultural co-evolution between farmers and their traditional varieties. Farmers do not want to loose their traditional varieties even if most of them also plant and try some new coconut hybrids. However, these conserved varieties must also be improved upon using a participatory approach so that the farmers' preferences are not lost during the selection process.

10.5.2.2 Inter-Varietal Breeding Strategies

In the case of coconut breeding, the term 'hybrid' is used by scientists in its widest sense. It is defined as a cross between two structures belonging to two different coconut varieties. The term 'structure' here means a variety, a population of palm selected from a particular variety, a family or an individual. For instance, a well known coconut hybrid is the cross between the Malayan Red Dwarf (MRD), an autogamous variety that is close to a single homozygous genotype, and the Tagnanan Tall (TAGT), an allogamous population containing palms with different genotypes but showing well identified similar traits.

The first known coconut hybrids were created in 1926 by Marechal, a scientist who crossed the MRD with the Niu Leka Dwarf in Fiji (Marechal, 1926).

Hybridisation between Dwarf and Tall coconut varieties were first done in India in 1938 (Patel, 1938). However, a recent study showed that the first coconut hybrids were discovered not by the scientists but by traditional farmers from Kerala (India). In these farmers' fields, Dwarf varieties were available until at least 1885 (Shortt, 1885). One of these Dwarf coconuts named Chowgat Orange Dwarf (COD) is mainly autogamous. When it reproduces 'true to type' by selfing, the colour of the sprout from new germinated seed-nuts is orange. But, up to 20% of the seed-nuts harvested on this Dwarf variety had brown-coloured sprout instead of orange (Thampan, 1998; Pramod et al., 2003). These off types are natural crosses between the COD and the surrounding Tall palms of green and brown colours (the latter being 95% from the variety named West Coast Tall). These brown seedlings have been popular among Indian farmers for a very long time. Farmers did not have scientific knowledge about the reproduction mode of the coconut palm, but they knew well how to select hybrids from their own nurseries.

The development of hybrids from the traditional varieties brought about considerable increases in copra yield (Fig. 10.3). The superiority of hybrids has been confirmed under a very wide range of soil and climatic conditions (Nuce de Lamothe and Rognon, 1986). Although some high yielding hybrids may not be adapted to some cultivation areas (for example, because of disease susceptibility), it is very likely that for each region, well adapted, high-yielding hybrids might be identified. A practical advantage of breeding hybrids is that the methods followed for seed production prevent self-pollination and thus ensure that the intended genetic progress is effectively transferred to the farmers.

There are three main types of coconut hybrids, ranked by order of economic importance: Dwarf × Tall (D × T), Tall × Tall (T × T) and Dwarf × Dwarf (D × D) hybrids. In scientific papers, hybrids are often designated by the international code of their parental varieties such as 'MYD × WAT'. The first variety code cited designates the female parent (MYD or Malayan Yellow Dwarf). Regulations about hybrids commercial names have yet to be established. For instance, the hybrid MYD × WAT bears the commercial designations of PB121 in Africa, MAWA in Asia except in Thailand where it is known as Sawi Hybrid No 1.

The first cycle of hybrid testing: Crosses between varieties are compared to a control, generally the local Tall cultivar or a well-known commercial coconut hybrid. Between the years 1940 and 1960, some coconut hybrids tests were carried out in various research centres, comparing the local varieties with D × T and T × T crosses made between local varieties (Bhaskaran and Leela, 1978). These trials in general involved a few palms per hybrid tested. Most of the results showed the superiority of hybrids above the local Tall varieties. However, the hybrids sometimes were considered to be too close to their Dwarf parents, as they were alternately bearing, subjected to bunch abortion and sensitive to drought. As the controlled pollination technique was not yet fully operational, the legitimacy rate of some of these hybrids needed confirmation. For a long time these experiments remained essentially theoretical due to the absence of an economically viable technique for seed-nut production to ensure wide distribution of the hybrids. Some countries turned away from this path of research as it did not seem to lead to practical applications.

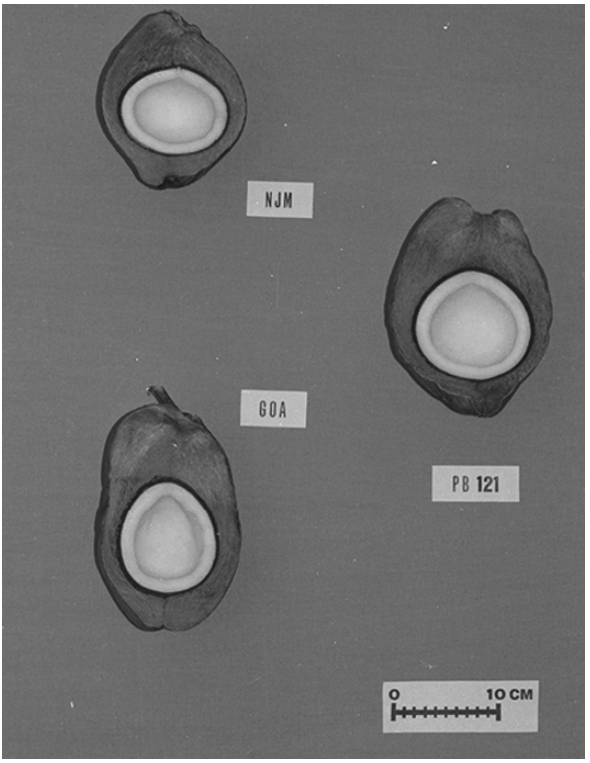

Fig. 10.3 The fruits of three coconut cultivars: The Malayan Yellow Dwarf (*top left*), the West African Tall (*bottom left*) and their hybrid: the PB121 (*right*) picture by R. Bourdeix (*See* Color Insert)

However, from 1960 to 1972, the development of new workable techniques made it possible to produce hybrids seed-nuts of good legitimacy at an economically acceptable cost (Nuce de Lamothe and Rognon, 1972a, b). This induced a new interest for coconut hybrids evaluation. There is no database for all the hybrids created and tested throughout the world. Nevertheless, based on inventory of the hybrids tested in the main breeding programmes, the total number of coconut hybrids tested worldwide was estimated to be more than 400 in 1993 (Bourdeix, 1999).

Improvement of the best hybrids: Some of the best D×T and T×T hybrids have been improved using the individual combining ability test method and by exploiting the genetic variability that exists within the populations of Talls (Gascon and De Nuce de Lamothe, 1976). For a description of this method, it is better to use an example. The hybrid PB121 is a cross between the MYD and a selected population of WAT. Its good performance in Côte d'Ivoire has stimulated its further improvement. Forty-five WAT parent palms were phenotypically selected and individually crossed with the same MYD population. The 45 progenies thus obtained are considered half-sib families. In only one generation of breeding, it was possible to improve

the yield of the earlier PB121 hybrid from 15 to 25%. Some of the improved F_1 progenies were also proven to be more tolerant to diseases. This method, initially developed by the Institut de recherche pour les huiles et oléagineux (IRHO, presently part of the Centre de cooperation intérnationale en recherche pour le développement: CIRAD) in the 1970s, was applied mainly in Côte d'Ivoire and Vanuatu on D × T and T × T hybrids using the West African, Rennell Island, Tahiti and Vanuatu population of Talls. The MATAG hybrid (MRD × Tagnanan Tall) is also being improved at the United Plantation Berhad, Malaysia.

Experiments conducted in Côte d'Ivoire have shown that by selecting 7–8% of the best families, a genetic gain ranging between 15 and 30%, depending on the trials, is achieved (Bourdeix et al., 1989). It confirmed the variability that exists within the natural population of the Tall. The analysis of these trials showed that phenotypic selection of parents is sometimes efficient but cannot substitute to the progeny test. Some good yielding genitors can have average combining ability value and inversely. Although the progeny test is costly, it is necessary for selecting the right parent palm. The best yielding progenies, in terms of copra per hectare, do not show above average number of bunches and copra content per nut. It seems that the genetic progress is mainly due to the improvement in the number of nuts per bunch. However, in some cases, the percentage of copra in the fruit improved slightly due to the reduction of the husk component of the fruit.

10.5.2.3 Breeding Prospects

Recurrent reciprocal selection: During the last four decades, most of the coconut breeding programmes have focused on the exploitation of hybrid vigour. However, the benefits from this option are not yet maximised; thus, there is a need for further refinement of the process. In fact, different methodologies can still be considered. Should one compare the progenies of populations, individuals, half-sib families or sib families? How should one structure the variability in order to maximise the genetic gain? The answers to these questions determine the future success of the coconut improvement programmes.

A balance of genetic programmes and trials was conducted by CIRAD in the 1990s. New directions were proposed for coconut genetic improvement (Bourdeix et al., 1990, 1991a,b). The proposed breeding scheme was based on reciprocal recurrent selection (RRS): creation of populations kept in reproductive isolation and improved with respect to each other (Comstock et al., 1949). The constraints related to reproductive biology of the plant, as well as other genetic factors, have led to the choice of the RRS method for half-sib families.

Two principal selection procedures have been described for the improvement of the D × T and the T × T hybrids. The procedure for the D × T selection is fairly simple. Dwarf and Tall coconut varieties show complementarities and good reciprocal combining ability. Hence the Tall population is improved in respect to the Dwarf population and this improvement is reciprocal for both.

The T × T procedure is more complex. A large part of the coconut variability is within the Tall varieties. Many T × T hybrids have shown a strong heterotic effect,

such as the crosses of WAT with the Rennell Island Tall, Vanuatu Tall or Thailand Tall. Attempt was made to classify the Tall varieties within two complementary groups based on their combining ability (Bourdeix, 1991a). Various attempts have also been made to structure the variability within the Tall varieties. These have led to divisions based on morphologic characteristics (Harries, 1978; N'Cho et al., 1993), enzyme markers (Benoit and Ghesquiere, 1984; Hartana et al., 1993; Fernando and Gamini, 1997), foliar polyphenols (Jay et al., 1989) or DNA markers (Ashburner et al., 1997; Lebrun et al., 1998a,b).

To be fully efficient, the RRS method requires the evaluation of at least 100 half-sib families per year. Including the hybridisation test, the selfing and the intercrossing of the parent palms, this would require planting each year of at least 100 ha of field experiments. This level of activity was reached in Côte d'Ivoire in the 1980s and may be in the Philippines in the 1990s. Currently, there is no research programme allocating such levels of resources to coconut breeding. It can be estimated that, on average during the period 2000–2005, less than 10 new coconut hybrids have been created yearly worldwide.

Complex hybrids: The term 'complex hybrids' refers to crosses of a coconut hybrid with another hybrid or a variety. In the case of coconut, the first complex hybrids were created by the Indian farmers of Kerala (Bourdeix et al., 2005, in press) (see inter-varietal breeding strategies). These farmers selected natural hybrids COD × WCT from the seed-nuts of Dwarf coconut palm. Then they harvested seed-nuts from the hybrid itself and planted it again. From this progeny, they again selected the brown-sprouted seed-nuts. Some generations later, they finally created a precocious and bright brown-fruited variety known as 'Komadan'.

For the coconut breeder, the first experiment testing of complex hybrids was established at the CNRA Marc Delorme Research Station, Côte d'Ivoire, in 1976. This programme essentially aimed at evaluating the genetic variability and searching for exceptional coconut palms that could be propagated rapidly when a method of vegetative propagation would become operational. This was quite ambitious and, some 30 years later, the development of the coconut in vitro propagation technique has not progressed sufficiently to be used for this purpose. Recently, however, Dr Carlos Oropeza reported at the Linkpalm biotechnology conference in Manila in 2002 that his group has improved the technology that is projected to generate 100,000 embryos from a single plumule, which is a substantial advancement in in vitro propagation of coconut.

In Thailand some three-way hybrids (D × T) × T and (T × T) × D were created in the 1900s to evaluate the genetic variability of these materials and to find out if these hybrids may be distributed to farmers (Petchpiroon and Thirakul 1994). To date, results of these experiments are yet to be published.

In Côte d'Ivoire, some of the crosses tested were (D × T) × (D × T), which gave some information about the inheritance of dwarfism. This study is in progress but its interpretation is constrained by the heterogeneous nature of the experimental plot. Dwarfism in coconut is a manifestation of many different characteristics such as precocity, autogamy, small size of organs and slow vertical growth. In a (D × T) × (D × T) progeny, the progenies do not look like a typical Dwarf. The resulting palms have a

wider bole and a faster vertical growth than the typical Dwarfs used as parents. They have an unexpected level of homogeneity. In the case of coconut, a single gene does not determine dwarfism. The corollary is the following: seed-nuts harvested from D × T hybrids do not give, at least in the first generation, a mix of Dwarfs, hybrids and Tall varieties. Many farmers indirectly produced complex hybrids and the result will probably be not as disastrous as predicted earlier by scientists, as shown in Table 10.4, even if the best F_1 hybrid remains superior to complex hybrids.

Complex hybrids between Dwarf varieties were also created in Côte d'Ivoire in order to find varieties tolerant to the Lethal Yellowing disease in Ghana (see section on breeding for secondary attributes).

No breeding programme went further than the second generation (D × T × D × T) or (D × D × D × D) since the 1990s except in the Philippines that aimed to create Makapuno Dwarf autogamous varieties from Makapuno Tall and normal Dwarf varieties (Nunez and de Paz, 2004).

Synthetic or composite variety: Breeding for a synthetic or a composite coconut variety (CCV) was first proposed in India and Sri Lanka. Subsequently, more attention was given to the development of CCV with the work of Santos (1990) and Santos and Rivera (1994) in the Philippines. Six Tall varieties from the Philippines and Africa were crossed to form 15 hybrids. These hybrids were planted together with the aim of producing a mixture of four-way hybrids by natural pollination. This mixture and the following generations from open-pollination were released to farmers as the CCV variety. In spite of its simplicity, this method presents both advantages and some disadvantages.

According to the genetic theory, the CCV will always be less productive than the best F_1 hybrid that could be made from the parental varieties of the CCV. Nevertheless, the F_1 hybrid needs to be produced in an expensive seed garden, while the CCV can be reproduced by open-pollination by the farmers themselves. Most

Table 10.4 Fruit production of two complex hybrids and a D × T hybrid in an experimental plot planted in 1987 in Côte d'Ivoire

			Average fruit production per palm per year (4–14 years period)	
Hybrid tested*	Type	Number of palms harvested	Mean	Standard Deviation**
PB121 or MYD × WAT	(D × T) Control	95	109	44
(MYD × WAT) × (BGDxRIT)	(D × T) × (D × T) Dwarfism segregation	348–339	94	49
(CRD × MYD) × (WAT × RIT)	(D × D) × (T × T) No dwarfism segregation	211–208	81	52

*MYD: Malayan Yellow Dwarf; WAT: West African Tall; BGD: Brazil Green Dwarf; CRD: Cameroon Red Dwarf; RIT: Rennell Island Tall.

**Mean of annual standard deviations.

farmers plant more than one coconut variety in their fields. Naturally, CCV palms will be surrounded and pollinated by neighbouring palms. Reproduction by farmers will progressively dilute the CCV variety that will loose its original value. On the other hand, from an evolution point of view, selection conducted by farmers could result in the emergence of new interesting types of coconut palms.

As the reproduction mode is not controlled during the process, selfing of CCV palms will occur that may result in strong inbreeding depression. First results of a recent molecular study indicate that the CCV was predominantly cross-pollinated with out-crossing rates of 91.1–91.4% and a large proportion of the cross-pollination events were due to full-sib mating. Therefore, the average selfing rate seems to be relatively low. However, selfing in the coconut palm is seasonal and depends on the speed of emission of the inflorescence. The faster the emission of inflorescences, the higher is the probability of selfing between successive inflorescences. CCV planted under bad agronomic management could have a low selfing rate while the same variety planted in good agronomic conditions will have a higher selfing rate. The practical solutions to restrict accidental selfing as suggested earlier (see the section on Mass Selection) could be used to reduce selfing rate in the open-pollination process of CCV.

10.6 Varietal Screening and Utilisation

10.6.1 Practical Methods for Coconut Hybrid Tests

COGENT has published a Manual on Standardized Research Techniques for Coconut Breeding or STANTECH (Santos et al., 1996) that is used as a guide by COGENT member countries in developing hybrids and conducting hybrid trials. This manual has enabled coconut breeders to use the same protocol to generate data that can be compared. Due to their differences in growth in height and width of the canopy, D × D, T × T and D × T hybrids are usually tested separately. The experimental designs used often are randomised complete or incomplete blocks, consisting of large plots: for instance, six lines of four palms or four lines of four palms. In addition to standard statistical considerations, two phenomena should be considered: competition between palms and xenia effects on fruit size. The various hybrids planted in a comparative trial rarely have the same rate of growth or height. The competition for light develops at the age of nine or ten years and influences the final yield evaluation. The genotype of the pollen also influences the weight of the triploid albumen (kernel) of the fruits. Large experimental plots make it possible to more accurately compare the palms at the inside of the plots (surrounded by the palms of the same genotype) which are buffered by the border palms that are in contact with different planting materials and more susceptible to border and xenia effects.

Usually, the production is distinguished between young age, from 2 to 8 years after planting and adult age from 9 to 12 years and older. Increasing the number

of years of observation should reduce the effects of biannual alternation. Fruits are harvested monthly (for fast germinating Dwarfs and D × D hybrids) or bi-monthly (for T×T and D×T hybrids). In many research centres, stealing of fruits are a critical problem that can strongly reduce the accuracy of evaluation. Hence, fruits per palm are sometimes counted before they reach the tender stage. The yield is traditionally expressed as weight of copra per palm or per hectare. The main recorded parameters are precocity of flowering, number of bunches and fruits, fruit weight and composition, vertical growth of the stem, resistance to pests, diseases and cyclones.

Throughout the world, the number of palms used to evaluate a hybrid has fluctuated from about 10 to over 140, depending on time and breeding programmes. Marshall and Brown (1973) have estimated that 60 to 100 palms could represent a natural population of allogamous plants. Due to the cross pollinating nature of the Talls, most of the palms within a Tall variety neither have the same genotype nor the same value in the cross. Thus the genetic homogeneity of a hybrid population is at least superior to that of its more variable parental varieties. The STANTECH manual recommends a minimum number of 96 palms per test hybrid.

CIRAD has developed practical methods for the controlled pollination process (Nuce de Lamothe et al., 1980). If the aim is to produce 96 'useful' palms of a given hybrid between two varieties then at least 48 palms of the variety to be used as female and 24 palms of the variety used as male will be required. In the experiment, border palms should be from the same hybrid variety. After planting, some palms may die and must be replaced the next year. Not all the seedlings from the harvested nuts will germinate. A controlled pollination sometimes yields as low as one seed-nut per bagged inflorescence. Therefore, the number of controlled pollination should be twice the number of desired 'useful' palms in the hybrid test, i.e 192 per hybrid. If the number of palms for female parents is 48, as they produce about one inflorescence per month per palm, the controlled pollination programme will take 4–5 months to be completed. Compared to assisted pollination that is used to produce the D × T hybrids, controlled pollination to produce T × T hybrids is a heavy and expensive task. Fruit setting is observed three months after pollination.

For the hybridisation tests, parental varieties are usually selected based on their proven performance in the areas of intended use, such as nut, copra or oil production. The other parent is usually chosen because it complements the specific weakness of the first parent, such as precocity or resistance to biotic and abiotic stress. Preferred major traits of a Tall parent are high productivity, broad adaptability and tolerance to specific pests and diseases. Dwarfs are preferred for their precocity and high rate of bunch emission (Bourdeix et al., 1998).

Most commercial hybrids are of the D × T type, which is easier to produce and allows combining the precocity of Dwarfs with the robustness of Talls. However, some T × T hybrids appear to have a comparable potential. They are more suitable as main crop for inter-cropping and offer better prospects for long-term genetic progress. So far, Thailand is the only known country where T × T hybrids have been released to farmers (Anupap et al., 1992).

D × D crosses are not very popular among coconut breeders except in Côte d'Ivoire where more than 40 crosses are being evaluated since 1993. Dwarfs are reputed to be sensitive to environmental stresses, such as drought and low fertility soils. Nevertheless, some of the most profitable coconut plantations in the world are probably those of Green Dwarf varieties found in Brazil and Thailand; with high planting density (more than 200 palms per hectare), high fertilisation rate and sufficient irrigation. The Thailand coconut breeding programme mainly focuses on the improvement of the Aromatic Green Dwarf varieties. An experiment was conducted in Côte d'Ivoire in 1971 to test the three possible hybrids between MYD, MRD and Brazil Green Dwarf (BGD) and compared them with MYD as control (Le Saint and Nuce de Lamothe, 1987). The hybrid MYD × MRD produced an average of 3.8 t copra per hectare that was comparable to the production level of a good D × T hybrid. An important feature of D × D hybrids is their high genetic homogeneity. As the two Dwarf parents are close to being pure lines, their progenies are less likely to be genetically variable than the D × T and T × D hybrids. A few promising D × D hybrids are also being currently evaluated at the United Plantation Berhad, Malaysia.

10.6.2 Multi-location Varietal Trial

As part of the national breeding programme, hybrid progenies are usually tested in various locations within the country using local cultivars as control before they are released as cultivars. Sometimes popular exotic varieties and hybrids are also included in the trials to determine their adaptability under local conditions. Among countries, results varied, but in general hybrids performed better than the local control. It is estimated that improvement of the best hybrids using individual combining ability tests can result in a productivity gain to the tune of 15–30 % in each new generation (Bourdeix et al., 1989).

In 2001, COGENT supported the Asia Pacific Coconut Community (APCC) in conducting a survey on the performance of coconut hybrids and varieties, and farmers' varietal preferences in 10 coconut producing countries. The results showed that: (1) there are no universal hybrids with each hybrid having their specific niches where it performs well; (2) hybrids performed better than traditional varieties under adequate rainfall and good soil conditions; (3) under optimum growing conditions and management, coconut hybrids tested could produce up to 5 t of copra/ha/year compared to the 2–3 tons obtained from traditional Tall varieties under good management level; and (4) farmers were not only interested in high yields per se but also in other characteristics such as low input-requiring varieties and varieties with special characteristics for producing high-value products.

Although the tested varieties/hybrids showed promising results their performance differed across countries. From 1999 to 2004, COGENT conducted a Common Fund for Commodities (CFC)-funded hybrid multi-location trial involving three African countries (Côte d'Ivoire, Benin and Tanzania) and three Latin American and

Caribbean countries (Brazil, Mexico and Jamaica) to verify if indeed the patterns of genetic differentiation are strongly related to environmental heterogeneity (Batugal and Benigno, 2005). Each of the six countries compared same six promising hybrids produced and shipped from Côte d'Ivoire with hybrids produced locally. The common imported hybrids were four D × T and two T × T hybrids with proven record of good yield potential in separate trials. Four to eight promising local hybrids/varieties were included in each trial to serve as local control by the respective country. The Government of Portugal funded a similar project involving the evaluation of the same six imported hybrids and four local hybrids in Mozambique. This brought the number of coconut hybrids being evaluated to 38, making this project the most comprehensive coconut hybrid trial worldwide.

The most important result of the project is the identification of 19 early bearing and high-yielding new coconut hybrids. Nineteen out of 34 coconut hybrids in the first trial started to flower and produce fruits in Brazil, Jamaica and Mexico in 2.5–3 years after planting compared to the 7 years it would normally take for the traditional Tall varieties to reach fruiting stage. In Brazil, two hybrids from Côte d'Ivoire and two local hybrids flowered; in Jamaica, all six hybrids produced in Côte d'Ivoire flowered but none of the local hybrids; while in Mexico, only one hybrid produced in Côte d'Ivoire and eight locally produced hybrids flowered. On the other hand, flowering was not observed in the hybrids planted in Benin, Côte d'Ivoire and Tanzania during the same period. These results suggest a possible germplasm × environment interaction but this could be verified with the vegetative and reproductive plant measurements and biotic and abiotic stress data to be gathered and analyzed in the next five years.

Based on the yield projection of the potential of the 19 fruiting hybrids in their fourth year, they have the potential to produce up to 5–6 tonnes of copra (dried kernel) per hectare per year at peak production (at 10–12 years) compared to the one metric tonne of copra produced by the traditional coconut varieties. The impact of the results from this CFC-funded project is significant as it has the potential to increase coconut yields of resource-poor smallholder coconut farmers five-fold. These hybrids and good production management are to be effectively promoted in many coconut growing communities and countries with similar growing conditions. Although the hybrids in the second trial are all growing well in the five countries (except Benin), the full potential of the hybrids can only be determined when they start to produce fruits three years after project termination.

10.6.3 Utilisation of Hybrids

Many of the D × T hybrids have yields of 80 to 100% more than local Tall varieties under medium to good management conditions. Many good T × T hybrids, as sturdy as the local Talls and involving them as parents, were also created. No more than 10% of these T × T hybrids have been tested outside research stations. No more 10 D × T and T × T hybrids were improved using individual progeny selection and

that was made only in Côte d'Ivoire and Vanuatu. These improved hybrids produced 15–25% more than the first generation of hybrids but these hybrids have yet to reach more farmers (Bourdeix et al., 2001).

Although hybrids generally performed better than the traditional varieties, they are currently being grown in limited areas, i.e. less than 0.1–14% of the coconut area in a country. It can be estimated that the hybrids represent $< 3\%$ of the 10–20 million coconut palms planted yearly worldwide. As previously mentioned, the poor adoption of hybrids may be attributed to inadequate information dissemination on the availability of improved hybrids/varieties and affordability of the planting materials. Those who planted hybrids mostly favoured D $\times$ T and T $\times$ D due to their high yield, early bearing, good nut size and better resistance to pests and diseases. However, some dissatisfaction on these hybrids was expressed in terms of bunch buckling (bunches giving way under weight pressure), high input requirement, vulnerability to moisture stress and pests and diseases (Batugal, 2004).

Disease and pest resistance is one of the main challenges of coconut breeding since many coconut growing countries are severely affected by disease-causing pathogens and harmful insects. Drought tolerance is also an important aspect needing attention, especially in agro-ecological zones where the rainfall is inadequate and irregular.

Improvement of special coconut varieties, such as the soft endosperm Makapuno in the Philippines, aromatic Dwarf in Thailand or the juicy Brazil Green Dwarf, is of significant economic importance. Some other varieties, such as those known as 'sweet husk' are still under-utilised although they have a high commercial potential.

10.6.4 Lethal Yellowing

Lethal Yellowing is a disease (or a complex of diseases) caused by phytoplasma that rapidly destroy large coconut areas in several regions. It appears to have been observed in the nineteenth century in the Cayman Islands, Cuba and Jamaica (Arellano and Oropeza, 1995) and has been present in the region since then with epidemic episodes, followed by quieter periods. Similar diseases are found in West Africa (Ghana, Togo and Cameroon) and in East Africa (Tanzania and Mozambique). This disease is also found in 30 other species (Thomas, 1979) including the date palm (*Phoenix dactylifera*) and ornamental palms (*Adonidia merrillii, Pritchardia thurstonii*), and thus the eradication of potential reservoirs for the disease is not a practical method. Treatment through injection of a tetracycline-type antibiotic (McCoy, 1972) is efficient but its high cost prevents its wider application.

A totally efficient control method still has to be developed to reduce the populations of vectors, but eradication of the first diseased palms (Dery and Philippe, 1995) and the adoption of suitable varieties could be used as pest management intervention. In effect, each of these components appear to be insufficient to control the disease by themselves, while there is some hope that a combination of components would be capable of reducing its virulence to an economically manageable level.

The genetic strategy was adopted in Jamaica where the Malayan Yellow Dwarf (MYD) and its hybrid with the Panama Tall (PNT) or Maypan were used extensively for replanting the affected regions in the 1980s. This strategy appeared to be successful until the beginning of this century when the disease destroyed extensively the supposedly resistant materials. MYD and PNT were chosen in an extensive series of trials (Been, 1981). The mapped tested cultivars are shown in Fig. 10.4. It is interesting to see that virtually all the cultivars that had some level of tolerance (below 35% infection) were from Southeast Asia. This is even more striking if we consider that, according to molecular criteria, the pre-Colombian Panama Tall and the Peru Tall are actually related to the Southeast Asian cultivars. This result is potentially important in the search of germplasm with improved resistance in the Asian region.

Various cultivars and hybrids were also tested in Ghana and the only two cultivars, which were not affected by the disease, were the Sri Lanka Green Dwarf and the Vanuatu Tall (Mariau et al., 1996). The latter was very susceptible in Jamaica. The MYD behaved rather well, but had a small percentage of diseased trees. Likewise, in Tanzania, Dwarfs seemed to behave better than the Talls, especially the Cameroon Red Dwarf and the Equatorial Guinean Dwarf (or Brazilian Green Dwarf). Research has also been conducted in the Yucatan Peninsula of Mexico, involving mainly Mexican material from the Atlantic and the Pacific coast of Mexico. The Atlantic Tall was highly susceptible while one group of Pacific Tall had a low level of susceptibility (Zizumbo Villarreal et al., 1999).

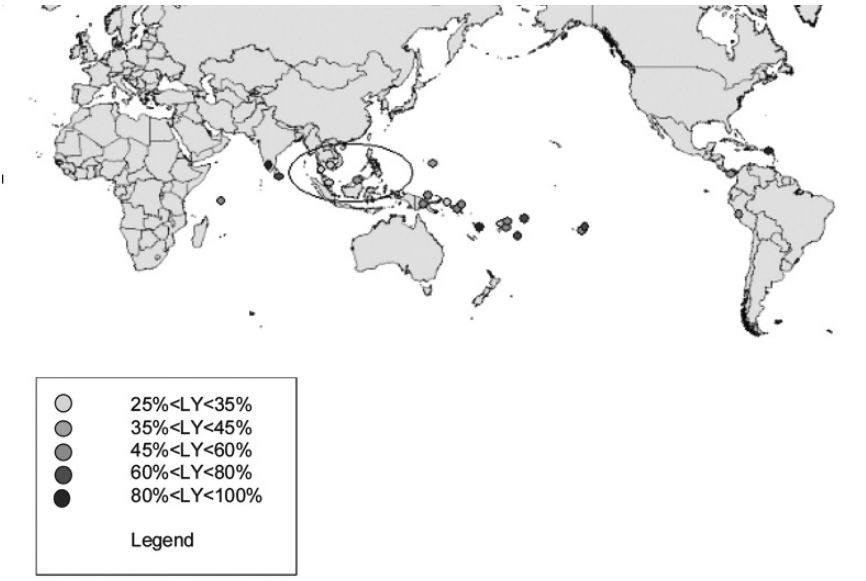

Fig. 10.4 Incidence of Lethal Yellowing disease on 25 Tall cultivars planted in Jamaica according to their geographical origins (Source: Been et al., 1981) (*See* Color Insert)

From the Jamaican experience, it seems unlikely that a single variety can be considered resistant in the long term. Moreover, the behaviour of the VTT suggests strong host-pathogen interactions. As a result, active search for a broader range of partially resistant cultivars remains necessary. Material from Southeast Asia and Mexico seems to offer the most promising material to date.

10.6.5 Breeding for Tolerance to the Phytophthora Diseases

Several species of *Phytophthora* cause major diseases of palms throughout the world. Lethal bud (heart) rot is the most common and devastating symptom of the disease. *P. palmivora* is the main coconut pathogen in South Asia. In Côte d'Ivoire, at the Marc Delorme Research Station, the main problem is caused by *P. katsurae* that causes premature nut fall and reduces yields by 10 to 80%, although it does not cause palm mortality. The same fungus has killed large numbers of coconut palms used for landscape in Hawaii. Till date, researchers have not succeeded in understanding why, in some cases, palms are dying while in other cases they are not; and the only visible symptom is nut drop.

The coconut hybrid PB 121 (MYD × WAT) adapts to a wide spectrum of situations, notably with good tolerance to water stress. Yet, the introduction of PB 121 in some regions of Indonesia and the Philippines ended in failure: *Phytophthora* killed a substantial number of palms in the plantations and farmers judged the nuts to be too small when palms are not fertilised. In the Philippines, geographical distribution of *Phytophthora* bud rot on coconut was studied from 1990 to 1999 through a survey in areas with reported incidence of the disease (San Juan Bachiller, 2004). Over 11,000 palms were killed by *P. palmivora* with Davao del Norte, Davao del Sur and Davao City having the highest incidence. The disease infected several coconut cultivars all over the country. Most of the affected palms were 3–15 years old with MYD or MRD parentage. The PB121 hybrid introduced into the country was the most susceptible, which was known to have a global incidence rate of 2.7%. Despite these claims of Concibido-Manohar (2004), this rate does not seem so high (in terms of yield) when the PB121 hybrid yields twice compared with the local Tall varieties during the first decade of bearing. Most of the problems were encountered only in very wet zones. Anyway, it pointed out that large scale plantation of a unique type of hybrid should preferably be avoided.

10.6.6 Breeding for Drought Tolerance

The coconut palm generally grows well in places where the annual rainfall is between 1,300 and 2,500 mm and above. An average monthly precipitation of 150 mm is generally considered ideal. A prolonged dry season lasting up to four months may adversely affect the palms; this kind of climatic aberration occurs

periodically in various important coconut growing zones, such as in Kerala (India), Sri Lanka or the West African coast. The adverse effects of drought on coconut may persist even for the subsequent 2–3 years. Hence, it is important to select coconut varieties which could overcome such drought periods.

Plant responses to drought are complex, varied and involve many different molecular, biochemical and physiological processes (Ingram and Bartel, 1996). Ollagnier et al. (1983) observed in Côte d'Ivoire and Indonesia that drought resistance seems to go hand-in-hand with resistance to leaf diseases and is also strongly influenced by the level of chlorine available in the soil.

Rajagopal et al. (1990) standardised the techniques on screening coconut varieties for drought tolerance using epicuticular wax, stomatal frequency and leaf water potential. Drought tolerance has also linked the relative level of lipid peroxidation (Chempakam et al., 1993). Coconut plantlets cultivated in vitro have been subjected to strong osmotic pressures with the objective to screen for drought-tolerant coconut germplasm (Karunaratne et al., 1999). These methods were mainly used to compare varieties but not individual coconut palms within a given variety (except the in vitro test). Although the results were interesting, there is need to compare results of drought resistance tests in vitro and in the field.

10.6.7 Breeding for Other Traits

10.6.7.1 The Makapuno Varieties

Although comparable varieties are known in many other countries, such as Sri Lanka, India, Cambodia, Thailand or Vanuatu, the Philippines' Makapuno is the most popular and economically important among the soft-endosperm coconut.

Growing Makapuno is unlike growing ordinary coconut trees. Its embryo germinates abnormally, as the soft endosperm – the main feature of Makapuno – grows extensively almost filling up the whole nut cavity. This abnormality is due to a single recessive gene that controls the Makapuno endosperm. Palms that could give potentially 100% Makapuno nuts were developed by Dr. Emerita de Guzman in the 1960s using coconut embryo culture techniques (De Guzman, 1970).

In 1996, Makapuno export from the Philippines was valued at US$ 2.5 million. Both local and export markets are expanding but supply fell short by about four million kg. As they are found in the Philippines only within the Laguna Tall variety, embryo cultured homozygous Makapuno palms from these heterozygous Makapuno-bearing sources are late bearers and highly cross-pollinated. At the National Coconut Research Centre in the Visayas four Dwarf $\times$ Makapuno crosses have been developed. Homozygous Makapuno palms of the second and third filial generations have been derived from those hybrids. The homozygous palms proved to be precocious, flowering at 26 months from planting. Self pollination was possible and Makapuno yield percentages were also high. The use of these crosses could therefore markedly increase farmers' income.

10.6.7.2 Aromatic and Other Varieties for Tender Nut Production

Due to its special aromatic flavour, the Aromatic Green Dwarf known as 'Pandan' in Malaysia produces one of the most appreciated coconuts for fresh beverage. Brazil Green Dwarf (BGD) is also renowned for its very sweet juice of tender nuts. With irrigation and fertilisation, BGD produces around 150 nuts/palm/hectare at a planting density of 200 palms per hectare and about 59,000 hectares of this cultivar are planted in Brazil. Both Aromatic and Brazil Green Dwarf are currently being improved in their respective countries.

On many Pacific islands and in Sri Lanka, rare coconut palms are reported that display very special characteristics. The husk of young fruit, which is usually bitter, tough and astringent, is tender, edible and sweet in some varieties in these islands. On ripening, the husk fibre are much finer and whitish. A sufficiently ripe fruit can be husked with bare hands, which is impossible with an ordinary coconut. These coconut palms are usually called 'sweet husk' or SWH. Various surveys have shown that while most islanders are familiar with these SWH coconut palms, the palms are becoming increasingly scarce. It is currently very difficult to obtain any of their seed-nuts as fruits of the rare coconut palms that do exist are eaten immature by children who love them. These varieties need to be propagated, conserved and improved as they could have economic importance in the future.

10.7 Biotechnology

Biotechnology can be used to improve coconut in several ways. By improving our knowledge of coconut diversity, molecular methods could help breeders effectively choose varieties to be used in a breeding programme to maximise heterosis and the chances of incorporating useful genes. It could also be used to identify markers for quantitative trait loci (QTLs), i.e. individual genes that contribute significantly to the diversity of a quantitative trait. Usually, this kind of study involves studying a segregating population formed by the progenies of a controlled cross. Finally, if a large number of inexpensive markers is available, association mapping can be performed in natural or breeding populations without the need for a precise knowledge of the pedigrees.

10.7.1 Molecular Methods

The first attempts to characterise coconut genetic diversity beyond the phenotypic aspects involved the consideration of biochemical products of genes like isozymes (Cardena et al., 1998; Hartana et al., 1993) and leaf polyphenols (Jay et al., 1991). However, in the last few years, most studies were devoted to the polymorphism of DNA markers.

In the last 10 years, a large number of studies were conducted using various methods to reveal DNA polymorphism. The general principles of these techniques are

described in Grivet and Noyer (1999). They involve purifying DNA and selecting fragments of variable length, which can be determined through gel electrophoresis. Their presence in a genotype is revealed by the presence of a stained (or radioactively labelled) band at a certain position on the gel. Some of these markers are dominant, i.e. they are detected by the presence or the absence of a single band, and homozygotes for the presence of the band cannot be distinguished from heterozygotes. The techniques include RAPD (Ashburner et al., 1997; Wadt et al., 1999), AFLP and ISTR (Duran et al., 1997; Rohde et al., 1995).

Other markers are co-dominant, since each allele corresponds to a different band and heterozygotes can be distinguished by the presence of two bands. This is the case in RFLP (Lebrun et al., 1998b) and in micro-satellites (Perera et al., 2000; Rivera et al., 1999). Although there are important differences between these markers in terms of cost, repeatability and degree of polymorphism, the picture they give of coconut diversity is very comparable (Teulat et al., 2000).

More recently, due to increased efficiency, high throughput methods like Diversity Arrays Technology (DArTs) (Jaccoud et al., 2001) are being tested in coconut under the 'generation' challenge programme. Its potential interest is to score several hundreds of dominant markers at a time and at a reasonable cost, thus making association mapping possible.

10.7.2 Determination of Genetic Variability

10.7.2.1 Importance of Genetic Diversity for Coconut Breeding

The raw material for plant or animal genetic improvement is genetic diversity. In coconut, a large array of morphological and agronomic traits is subject to genetic variation, including vegetative development, adaptation to biotic and abiotic stresses and, of course, nut yield and fruit shape and size. This is also the case of traits related to the quality of the product like tender nut water and albumen oil contents and processing quality. Part of this wide genetic diversity is accounted for by the variability of ecological conditions prevailing in the regions where coconut is grown, but human factors also play a significant role. First, farmers have tended to propagate or protect preferentially palms that best meet their needs; second, they took coconuts, along with other crops, with them during their migrations. The result was the creation of numerous populations originating from a small number of individuals (Bourdeix et al., 2001), a circumstance that strongly favours genetic differentiation. As a result, more than 300 named coconut cultivars have been identified across the world and represent a valuable reservoir of variable genetic diversity for plant breeders. For the most important ones, their phenotypic characteristics are well described (Baudouin et al., 2000).

Efforts to quantify genetic diversity in coconut pre-dated the advent of molecular marker. However, phenotypic studies as performed by N'Cho et al. (1993) give an incomplete view of genetic diversity because the considered traits are influenced by

environment and similar phenotypic features found in different populations result from different combinations of genetic factors.

10.7.2.2 Micro-satellite Kit for Coconut Identification

Among the available systems, micro-satellites were chosen to develop a kit for identifying coconut cultivars. In effect, its co-dominant nature and the large number of alleles make it possible to reveal a large spectrum of diversity in coconut. Moreover, such markers are revealed through the PCR technique, which is relatively inexpensive and less demanding in terms of DNA quality than others. This kit (Baudouin and Lebrun, 2002) comprises five components:

1. The sequences of 14 primer pairs corresponding to the 14 micro-satellite loci;
2. A set of more than 100 coconut reference populations from the whole coconut cultivation area and represented by about 600 individuals. Since the initial release of the kit and as a result of further studies, the number of individuals has increased to more than 1,000;
3. Standard DNA sequences from known coconut genotypes. These sequences are used as a means to determine the absolute length of the micro-satellite sequences;
4. Identification software. Geneclass2 has been designated to assign individuals or groups of individuals to their likely origin. Documentation and manuals. The need for specific software arises from the cross-pollinating nature of most coconut cultivars. In effect, in such populations, all individuals are different but most alleles are common to a large number of populations. As a result, probability calculation is required to assign individuals to populations based on allele frequencies. Allele frequencies are known only from inspection of a limited number of individual genotypes and the resulting uncertainty can be dealt with using Bayesian methods (Baudouin and Lebrun, 2000; Baudouin et al., 2004; Piry et al., 2004). Combined with the results of more classical methods like classification analyses (dendrograms) and multi-variate methods like factorial analyses, application of Geneclass2 to the reference populations made it possible to improve our knowledge of coconut diversity. When available, historical information on human migrations and on the movements of coconut was also taken into account; and
5. Outline of genetic diversity in coconut. Coconut cultivar diversity can be represented by a three-level classification (Baudouin and Lebrun, 2002).

The main division is represented by two main groups (Fig. 10.5). The Pacific group (A) is the largest and the most diverse. It extends from Southeast Asia to the South Pacific and the West coast of the American continent and includes both Dwarfs and Tall cultivars. The other group originated in South Asia but its present distribution covers the largest parts of the tropical coastal areas of the Indian and Atlantic Ocean. It is thus called Indo-Atlantic (B). At this level groups are clearly distinguished by the presence or absence of a few characteristic alleles. In contrast, further division represents geographical variations whose frontiers are not precisely

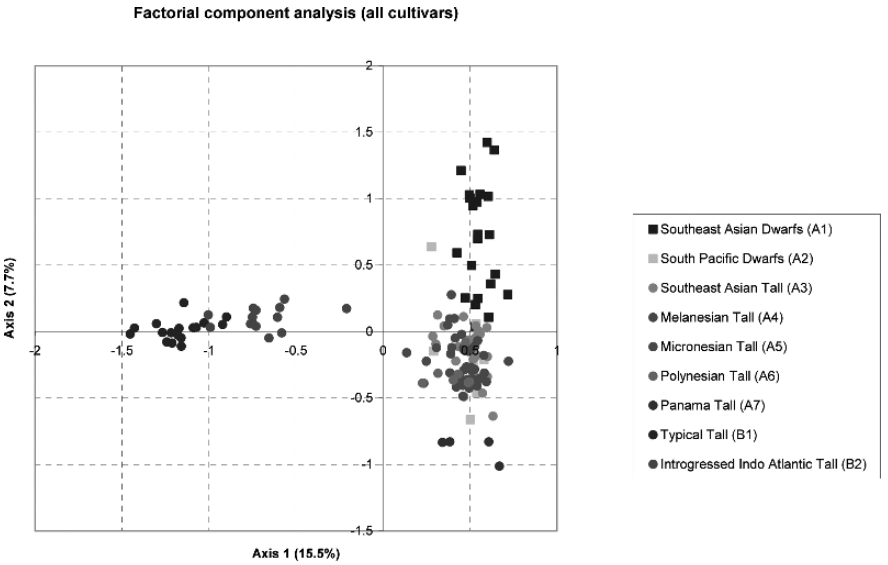

Fig. 10.5 Factorial component analysis of micro-satellite diversity using all populations (*See* Color Insert)

established. Their distinction relies on the variation of allele frequencies at several loci rather than on the presence of characteristic alleles.

All Dwarfs belong to group A irrespective of the place where they are found presently. They can be easily identified due to their self-pollinating habit; heterozygote Dwarfs are exceptional. Most Dwarfs come from Southeast Asia and have many common features at the molecular level. They can be further divided into a Malayan type (A1a) and a Filipino type (A1b). There are also a certain number of Dwarfs in the South Pacific. Their molecular traits are more variable and they form a separate group (A2).

Pacific Tall coconuts form five groups at level 2 on a geographical basis (Fig. 10.6). The Southeast Asian cultivars (A3) can be further divided into Continental (A3a), Indonesian (A3b) and Filipino (A3c) types. Diversity in the Melanesian group (A4) is high within cultivar as well as between cultivars. Five types can be distinguished using micro-satellites: the first two correspond respectively to the north (A4a) and the south (A4b) of New Guinea. The third type (A4c) is scattered on the smaller islands of Papua New Guinea. The Markham Valley Tall is very distinct and forms a type of its own (A4d). Finally, cultivars from Vanuatu, Solomon Island and New Caledonia form the fifth (A4e).

The relatively small number of cultivars studied so far in Micronesia (A5) and Polynesia (A6) does not justify further division at the third level. Cultivars from Panama and Peru form the last subdivision (A7) in the Pacific group. It is important to note that this group, which is probably pre-Columbian, differs from cultivars found on the Pacific coast of Mexico. The latter belong to group A3c and are the result of importation from the Philippines by the Spaniards.

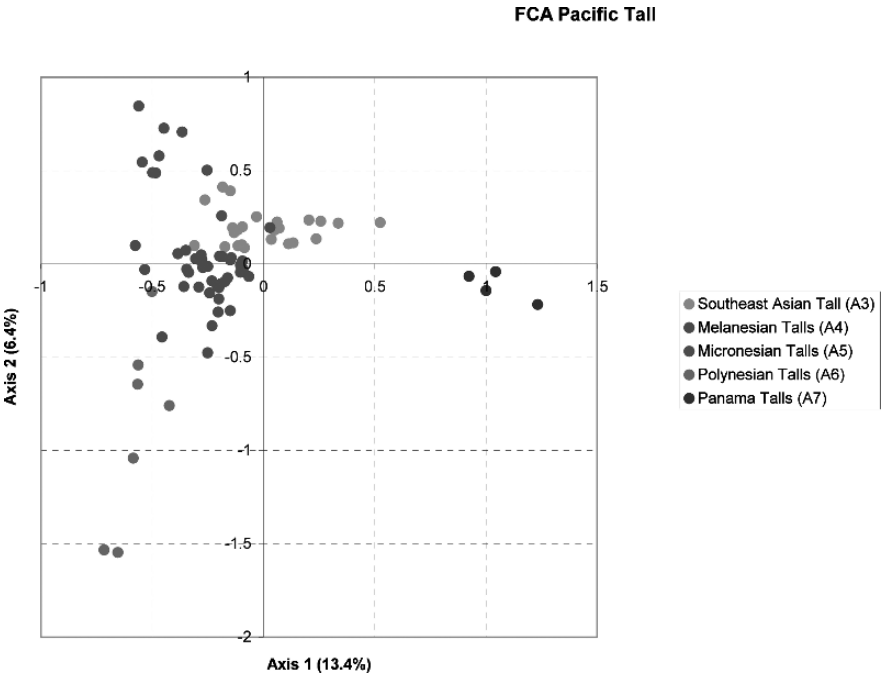

Fig. 10.6 Factorial analysis of correspondences on Pacific Talls (for clarity only level 2 groups are shown) (*See* Color Insert)

Diversity in the Indo-Atlantic group is lower than in the Pacific. It is however possible to distinguish two sub-groups (Fig. 10.5): the first is found in South Asia, West Africa, Latin America and the Caribbean region. It probably resembles the original populations, which developed in South Asia and is thus called 'typical Indo-Atlantic' (B1). The other sub-group or 'introgressed Indo-Atlantic' (B2) is found in East Africa, the Andamans and is also represented in South Asia. In addition to the typical genes of group B, they have a certain proportion of alleles that are otherwise found only in group A (about 23% in average). Introgression in East Africa is due to past migrations of populations from Southeast Asia to Madagascar. These populations certainly brought coconut with them (Lebrun et al., 1998a).

10.7.3 Practical Applications

10.7.3.1 Securing Germplasm Quality and Availability

The above-described methods are useful to the breeder by allowing him to conserve germplasm more economically. Conserving coconut genetic diversity is very costly since coconut seeds are recalcitrant (they cannot be stored as seeds) and half an hectare is necessary to preserve the diversity of a cultivar (Santos et al., 1996).

Avoiding unnecessary duplication is thus important. For example, Comparing 10 Brazilian Tall populations, it was possible to form 2 groups of 3 populations thus reducing the required area by 40% (Ribeiro, unpublished).

Other applications in this field are to assess the representativeness of a collection relative to the global coconut diversity and to identify priorities for germplasm acquisition and/or exchanges. These methods may also be used to check for possible genetic contaminations and loss of diversity during rejuvenation.

Finally, coconut populations in farmers' fields are an important resource for coconut improvement. On-farm conservation and participatory breeding rely in large part on spotting populations presenting original features, and molecular characterisation is important for that purpose. Thus, COGENT has initiated characterisation of farmers' varieties using farmers' protocol and molecular markers in eight Asia-Pacific countries.

10.7.3.2 Breeding and Seed Production

The molecular based classification together with phenotypic assessment outlined here may be used as a guide for choosing cultivars to be inter-crossed for breeding. In fact, genetic distance is directly related to the probability of heterozygosity in the resulting progenies. The benefit of such heterozygosity can be exploited in two steps: When two genetically distant varieties are crossed, hybrid vigour or heterosis is often observed. Hybrid vigour has long been recognised in coconut and serves as the basis of improved hybrid Dwarf $\times$ Tall production (Sangaré et al., 1988). Heterosis is however also found in Tall $\times$ Tall cultivars and, comparing the results of five hybrid trials, heterosis was at its maximum in hybrids between cultivars from the South Pacific (groups A4–A7) and group B1 ($+75\%$ in average). It was less marked ($+40\%$ in average) in other Tall $\times$ Tall combinations. This illustrates that, to a certain extent, genetic distance can be used to predict heterosis.

For the longer term, recurrent and recurrent reciprocal selection aim at fixing favourable allele combinations from different origins in order to obtain genotypes that surpass the parental populations. The chances of observing such transgressive genotype combinations are maximised if the parents of the original cross are genetically distant. This process can be made more efficient by the use of marker assisted selection. One additional advantage of wide crosses is maximised heterozygosity that increases the number of polymorphic markers in a mapping population.

The discussion about the distribution of genetic resistance factors to the form of Lethal Yellowing disease prevailing in Jamaica illustrates a different strategy – by studying the genetic origin of the cultivars that presented resistance factors to LY, it was possible to identify Southeast Asia as a probable source of resistance genes. The apparent exceptions of Panama Tall and Pacific Mexico Tall actually validates this hypothesis, since, according to molecular studies, they are related to Southeast Asian cultivars. This result can be used to diversify the sources of resistance in order to reduce the chances for the pathogen to overcome it.

Finally, investment made on coconut genetic improvement is costly and could be wasted if the progenitors used to reproduce selected crosses or populations were not

of the required genotype. This problem could be avoided with the use of the microsatellite kit. In addition, the technology has also been proven efficient in identifying off-types in seed gardens.

10.7.4 Molecular Breeding

10.7.4.1 Linkage Mapping and QTLs

As discussed in the previous section, molecular markers can be used as a tool for germplasm management and in choosing crosses that are expected to maximise genetic diversity and heterosis. They can also be used in a more direct way through marker- assisted selection as follows:

When ordered on a linkage map, anonymous markers can be used to identify chromosome regions where important quantitative trait loci (QTL) are located. Typically, the precision of the location is about ±10 centimorgans (cM). Various strategies are available to exploit this knowledge:

- Genotype building programmes through assembling of the favourable alleles of many QTLs in a single genotype;
- Introgression programmes are used to introduce the favourable allele of a specific QTL into an otherwise good variety; and
- Recurrent selection programmes use molecular score in addition to phenotypic data to predict the genitor's value.

Dekkers and Hospital (2001) made a comprehensive review of the different options for molecular breeding and of their prospects.

Efficiency in molecular breeding is at its best with traits exhibiting a moderate heritability; insufficient genetic variance in the targeted trait would prevent the identification of QTLs. On the other hand, conventional breeding would give satisfactory results with highly heritable traits. As genotyping is still expensive, the cost/benefit ratio of molecular breeding needs to be considered and situations that are favourable to the use of molecular breeding are mainly those where the target trait is difficult to assess (or cannot be assessed at each breeding cycle) like disease resistance, or where the measurement is expensive or time consuming. In the case of coconut, most of the traits related to nut production and quality require several years of observation after sexual maturity. The main benefits of marker-assisted selection in coconut result from saving time (e.g. marker-based selection at an early stage) or space (e.g. two-stage selection associating marker-based selection in nursery and trait measurement in the field for the selected genotypes only).

Several segregating populations have been used to construct linkage maps and to identify putative QTL markers: an initial coconut linkage map was presented by Rohde et al. (1999), used a cross between a Laguna Tall genotype from the Philippines and a Malayan Yellow Dwarf to construct a linkage map and to identify QTLs for germination precocity. The first linkage map involving an adult population

was presented by Lebrun et al., 2001) that allowed the identification of QTLs for yield components in a Cameroon Red Dwarf × Rennell Island Tall cross.

A recent study was devoted to QTL analysis of fruit component traits in the same population (Baudouin et al., 2005). A total of 52 putative QTLs were identified for the 11 traits under study. However, 34 of them were, in fact, grouped in 6 clusters, which probably corresponded to single pleiotropic genes. Some additional QTLs located apart from these clusters also had relative large effects on individual traits. QTLs for fruit component weight, endosperm humidity and fruit production were found at different locations in the genome suggesting that efficient marker-assisted selection for yield can be achieved by selecting QTLs for the individual components.

10.7.5 Alternative Mapping Populations

The cited results are promising, but the usefulness of linkage maps based on F_1 hybrid crosses is somewhat limited for two reasons: they exploit only a part of the existing genetic diversity – the one which is related to within-cultivar polymorphism and the chances of observing segregation at the same QTL in another cross are at the most 50%. Actually, a large part of *Cocos nucifera*'s genetic diversity results from the difference between Pacific and Indo-Atlantic cultivars. Thus, a special crossing plan (Fig. 10.7) was devised to identify in priority the QTLs that account for the differences between the two groups. Using such a design will result in more QTL's identified due to the choice of genetically distant parental populations. Moreover, this type of cross can detect QTLs that are fixed in the parental populations, which is impossible with conventional mapping populations. Such QTLs are easier to use in practical breeding. Using a Dwarf as tester makes it possible to produce a large number of progenies while simplifying the mapping task due to its homozygous genetic structure.

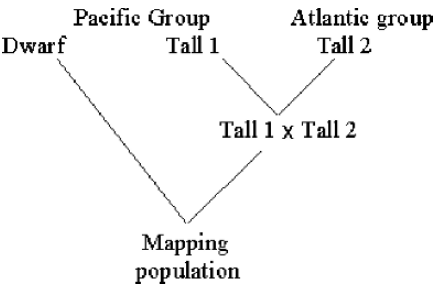

Fig. 10.7 A genetic design adapted to identifying QTLs in coconut

10.7.6 Association Mapping

Due to the low prolificacy of coconut and its bulkiness, large populations from a single controlled cross are rare and their production takes time. Another approach would be to correlate quantitative traits and markers in natural populations. This requires high throughput marker systems that are becoming more widely available (Jaccoud et al., 2001). It also requires larger populations, but the constraint due to controlled pollination is lifted. The proposed method called 'association mapping' involves the search for preferential association between closely linked markers (also called 'linkage disequilibrium'). As a preliminary step to association mapping, evaluation of the intensity of linkage disequilibrium will be examined by CIRAD researchers in Vanuatu.

Considering the number of traits involved in coconut breeding, the most satisfying way of improving traits could be the introgression of small number of favourable genes into a local cultivar through repeated back-crossing assisted by markers.

10.7.7 Embryo Culture and Safe Movement of Germplasm

Two of the major priorities of COGENT are saving threatened germplasm and promoting the use of conserved materials for developing improved varieties in coconut growing countries. Thus, accelerated effort is being placed on the movement of germplasm from COGENT's member countries to their respective regional ICG for conservation and multiplication and on the provision of breeding materials from the established ICGs to member countries. However, mass production of seed-nuts could not be achieved easily as there is a frequent allogamy responsible for high variability, a very long breeding cycle (at least 12 years), a low co-efficient of reproduction (100–200 seeds/palm/year) and lack of natural vegetative propagation (Hocher et al., 1999). Distribution of seed-nuts is also a major problem because of their big size, making transport cost prohibitive, their being recalcitrant (without dormancy phase) and due to phytosanitary restrictions of recipient countries. Alternatively, the more efficient way of facilitating access and safe movement of the coconut germplasm is through embryo culture. It is possible to delay the germination of zygotic embryos in vitro for one year (Assy Bah and Engelmann, 1993), which overcomes the recalcitrant nature of the seed-nuts.

Using in vitro techniques for collecting, exchanging and conserving coconut germplasm requires efficient protocols for in vitro germination and development of embryos into whole plantlets and for their acclimatisation to in vivo conditions and further development into plants that can be transferred to the field (Engelmann and Batugal, 2002). There were various protocols developed for coconut embryo culture in several laboratories worldwide (Batugal and Engelmann, 1998). Among these protocols, the most promising seem to be those developed by the University of Philippines at Los Baños (UPLB), India's Central Plantations Crop Institute (CPCRI), the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (IRD/CIRAD) and Institut des Forêts (CP/IDEFOR) of France and the Philippine

Coconut Authority-Albay Research Station (PCA-ARC) (Engelmann et al., 2002). These protocols are currently being tested and further improved by the COGENT member countries to suit their prevailing local conditions. Initial reports from participating countries showed that success rates have improved, i.e. from 14–55% to 31–80% of inoculated embryos developing into plantlets in vivo.

Currently and up to year 2010, the ICG host countries aim to conserve in respective regional field genebanks a maximum of 200 accessions each, which will be contributed by coconut-producing countries in each region (Batugal, 2004b). Related to this, COGENT published a germplasm health management manual on the operational management of germplasm movement involving seed-nuts, embryos and pollen (Ikin and Batugal, 2004). It is hoped that this manual will facilitate and accelerate the sharing of germplasm for coconut breeding.

In terms of long term conservation of the coconut germplasm in vitro, a working protocol for efficient cryopreservation of embryo (i.e. storage at ultra-low temperature, −196°C, in liquid nitrogen) is being developed. There is a need to refine and standardise the current technique, using various genotypes on a large scale in a genebank context (Engelmann, 1999). COGENT is currently promoting this initiative.

In vitro techniques have also been investigated in the last 30 years for vegetative multiplication through somatic embryogenesis (Hornung and Verdeil 1999). The aim was to exploit genetic diversity existing in a cultivar by cloning proven elite trees on a large scale. For this purpose, young leaf or inflorescence tissues of adult trees were chosen as explants. This approach proved to be very difficult and only a few plants were regenerated. Production of somatic embryos and their subsequent multiplication were difficult to obtain and finally, the regeneration of entire plants and their acclimatisation was also difficult.

Recent works concentrate on the use of plumule explants extracted from a zygotic embryo. Production and multiplication of somatic embryos is much more efficient (Pérez-Núñez et al. 2005). But this approach prevents assessing the ortet prior to sampling and this requires a further step in clonal selection: first, nuts are collected from a high producing palm to produce several clones and then these clones are tested in the field and the best ones are released.

In the meantime, regeneration and acclimatisation progress slowly. Tens of ramets are being planted in Mexico (Verdeil, com. Pers.). For the medium term, the main interest in clonal propagation is on the conduct of multiclon disease resistance trials. Replication would give breeders much more confidence about the status of resistant genotypes and would allow the search for QTL markers. Another potential application is reproducing progeny tested individuals to produce ‘clonal seeds’ that is, seeds produced with pollen collected on clones.

10.8 Conclusions and Recommendations

In the past, many coconut producing countries tried to improve varieties and developed hybrids for high yields, although they were limited by the lack of a wide range of diversity in their breeding programmes. With the involvement of several

development organisations which provided funding, germplasm and technical backstopping, many national breeding programmes have attempted to expand their breeding objectives to include adaptation and resistance to biotic and abiotic stresses. These breeding initiatives have been further enhanced through the establishment of COGENT that provided an effective mechanism for improving techniques and strategies for breeding and conservation and a platform for the exchange of germplasm and for collaboration on regional and inter-regional coconut conservation and breeding.

Precious coconut germplasm are still threatened by genetic erosion due to serious pests and diseases, typhoons and drought which kill them and man-made activities which compete for land on which coconuts are grown, i.e. urbanisation, housing, commercial land use, road construction, etc. These activities hasten the loss of important coconut diversity that is needed to produce improved varieties. The capacity building in existing national and international genebanks should be continued to sustain a comprehensive conservation and breeding programme. This capacity building is a major activity of COGENT in the next five years in collaboration with its partner institutions like CIRAD, the Asian and Pacific Coconut Community (APCC) and others.

National coconut R&D programmes should go beyond improving varieties. Emphasis should be given to ensuring that the good hybrids already available will be produced, evaluated and planted by coconut farmers. More attention should be paid to the questions of diffusion of coconut hybrids, acceptability versus availability of hybrids, economical and anthropological aspect of coconut seed-nuts, cultural co-evolution between farmers and their coconut varieties, and markets. Adoption of hybrids or varieties produced in breeding programmes is largely determined by their performance as judged by coconut farmer producers and other users. Thus it is important that coconut breeders exert more effort to fully understand the basis of varietal preferences of farmers and other users and consider these factors in planning and implementing their coconut breeding programmes.

COGENT will continue to address the above-mentioned issues in collaboration with national programmes, partner organisations, coconut farmers and NGOs through the following priority research in coconut breeding: (1) testing the best hybrids already identified out of the research centres using a farmer's participatory approach; (2) strengthening the diffusion of information to farmers especially by publishing catalogues containing reliable information on both traditional and hybrids varieties; (3) focusing breeding activities on important characters other than yield, such as tolerance to disease and adverse growth conditions, slow vertical growth, resistance to cyclones and other traits required by coconut stakeholders; and (4) providing a platform to narrow the technology gap between the research stations and farmers' fields. The latter strategy should focus on establishing an effective and sustainable system of propagating and distributing recommended hybrids, identifying the most suitable ecosystems where they will perform best and on technology transfer of appropriate cultural management techniques to achieve the desired socio-economic and environmental impact. COGENT is currently working on these strategies through capacity building and promotion of research collaboration among

its 38 coconut producing member countries and advanced laboratories worldwide. However, for these strategies to be sustainable, it will require more country commitments in terms of funding and human resources.

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Chapter 11
Opportunities for the Oil Palm via Breeding and Biotechnology

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11.1 Origin of Oil Palm

Historical records show that palm oil was initially traded for culinary purposes over 5,000 years ago (Zeven 1967). The oil palm (*Elaeis guineensis*) originated in West Africa, where it played an important role at the village scale for many centuries (Hardon, Rao and Rajanaidu 1985). Nevertheless, the exact centre of origin within Africa is not known (Corley 1976). In Southeast Asia, where it is one of the most important commercial crops (particularly in Malaysia and Indonesia), it has a rather short history. Four oil palm seedlings were brought from Africa via Mauritius and Amsterdam and planted in Bogor Botanical Gardens in Java (Indonesia) in 1848 (Fig. 11.1). The seedlings were subsequently distributed to Deli, Sumatra (Indonesia) in the 1870s (Whitmore 1973). In Malaysia, up to the late nineteenth century, oil palm was grown as ornamentals. In 1911 and 1912, palms of Deli origin were planted along the avenue in Rantau Panjang in the state of Selangor. Seedlings from the Rantau Panjang palms were planted in TenammaranEstate in 1917 and in Elmina Estate in 1920 in the state of Selangor, which marked the beginning of commercial oil palm cultivation in the country (Kushairi 1992). Ironically Malaysia's foray into commercial planting of oil palm owes its beginning to unsuccessful coffee (*Coffea sp)* cultivation at the Tenammaran Estate due to disease and drop in the price of the commodity in the global market (Jagoe 1952). The switch from coffee to oil palm led to the beginning of the commercial cultivation of the latter in Malaysia. Growth of the Malaysian oil palm industry was initially slow, gaining momentum only in the early 1960s when the Government introduced the diversification policy to ease dependence on rubber (*Hevea brasiliensis*), the major crop then.

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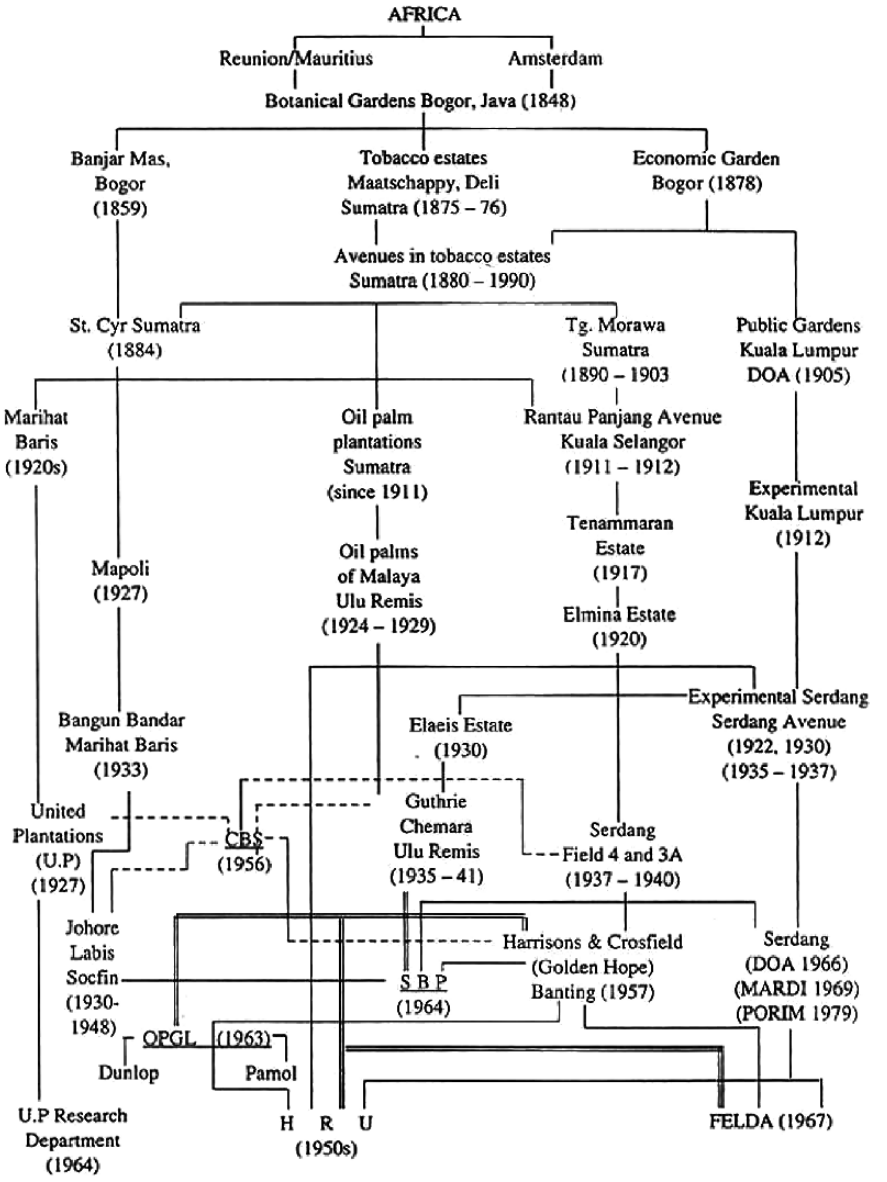

AFRICA
Reunion/Mauritius
Amsterdam
Botanical Gardens Bogor, Java (1848)
Banjar Mas, Bogor (1859)
Tobacco estates Maatschappy, Deli Sumatra (1875 – 76)
Economic Garden Bogor (1878)
Avenues in tobacco estates Sumatra (1880 – 1990)
St. Cyr Sumatra (1884)
Tg. Morawa Sumatra (1890 – 1903
Public Gardens Kuala Lumpur DOA (1905)
Marihat Baris (1920s)
Oil palm plantations Sumatra (since 1911)
Rantau Panjang Avenue Kuala Selangor (1911 – 1912)
Experimental Kuala Lumpur (1912)
Tenammaran Estate (1917)
Mapoli (1927)
Oil palms of Malaya Ulu Remis (1924 – 1929)
Elmina Estate (1920)
Experimental Serdang Serdang Avenue (1922, 1930) (1935 – 1937)
Bangun Bandar Marihat Baris (1933)
Elaeis Estate (1930)
United Plantations (U.P) (1927)
CBS (1956)
Guthrie Chemara Ulu Remis (1935 – 41)
Serdang Field 4 and 3A (1937 – 1940)
Johore Labis Socfin (1930-1948)
Harrisons & Crosfield (Golden Hope) Banting (1957)
Serdang (DOA 1966) (MARDI 1969) (PORIM 1979)
S B P (1964)
OPGL (1963)
Dunlop
Pamol
U.P Research Department (1964)
H R U (1950s)
FELDA (1967)

Fig. 11.1 History and development of the Deli dura in Indonesia and Malaysia till 1979
Source: Kushairi and Rajanaidu 2000.

11.2 Biology of the Oil Palm

A comprehensive description of the oil palm is provided by Purseglove (1972) and Hartley (1988). Oil palm has an unbranched stem topped by 35–60 pinnate fronds. It can grow to a height of over 20 m, producing about 2 to 3 fronds each month with each frond consisting of a petiole rachis and leaflets. The petiole is about 150 cm long and 300–400 leaflets are attached to the rachis. The palm reaches maturity when it is two to three years old. Oil palm can live up to more than 100 years, but its economic life does not extend beyond 35 years. The original palms planted in Bogor in 1848 were uprooted by strong wind force in 1992. The trees were 144 years old and would have survived longer if they had not been uprooted.

The oil palm is monoecious (male and female flowers produced separately on the same palm). The flowers are borne on spikelets that form the inflorescence. However, only either sex matures at any one time ensuring cross pollination. Duration of the floral cycle fluctuates between four and six months, influenced by genetic and environmental factors (Purseglove 1972). The inflorescence is a compound spike carried on a stout peduncle of 30–45 cm in length (Hartley 1988). Male and female inflorescence can have more than 100 spikelets (Rajanaidu et al. 2000). The female inflorescence has about 30 flowers per spikelet compared to the male inflorescence that can carry up to 1,200 flowers per spikelet. The flowers are bisexual in origin, but in the males the stigmas are suppressed while in the female flowers the stamens are underdeveloped (Hartley 1988). Hermaphrodite inflorescences are common in young palms and during the transitional phase of the floral cycle.

Sex ratio – proportion of female to total inflorescences – is an important yield determinant. High sex ratio, which is influenced by both genetics and environment, is desirable as it indicates higher yield attainment. Young palms can have a sex ratio as high as 98% and decreases to 35% in older palms (Latiff 2000). Other environmental factors that influence sex ratio include fertiliser application, planting densities and availability of sufficient water (Broekmans 1957; Corley 1977; Latiff 2000). The male inflorescence is known to produce a large quantity of pollen (30–40 g for each male inflorescence) (Rajanaidu et al. 2000). Kushairi and Rajanaidu (2000) reported that even up to 100 g of pollen could be produced per male inflorencence. Pollen is usually shed within five days after anthesis, and remains viable for five to six days. Oil palm pollen can be stored for up to 1 year at -5°C for subsequent use in breeding programmes (Hardon and Davies 1969). Pollen is usually dispersed by the weevil *Elaeidobius kamerunicus* and to some extent by wind. *E. kamerunicus* was introduced from West Africa to Malaysia in 1981 (Syed et al. 1982). Female flowers are receptive for 36–48 hours after anthesis during which time the stigma lobes are well separated, exude moisture and remain pink to trap the pollen grains (Latiff 2000).

Oil palm fruits are ovoid or elongated, 2–5 cm in length, weighing 5–20 g and tightly packed on fruit bunches. Each fresh fruit bunch (FFB) weighs about 10–30 kg and consists of about 1,500 fruits (Hartley 1988). Bunches exceeding 80 kg have also been observed. The oil palm fruit is a drupe, consisting of epicarp, mesocarp, endocarp (shell) and endosperm (kernel). The mesocarp produces palm

oil, while the kernel produces palm kernel oil, both of which differ in their fatty acid composition (FAC).

Besides shell thickness, oil palm fruits vary considerably in external appearance. Most notably is the epicarp skin colour – *nigrescens, virescens* and *albescens*. The most common is *nigrescens* – deep violet to black at the apex and colourless at the base before ripening (Hartley 1988). N*igrescens* fruit varies to some extent on ripening to either entirely red or black over the upper half but red at the base (Hartley 1988). *Virescens* – green when unripe and reddish orange when ripe. *Albescens* (which is rare) – deep green when unripe and pale yellow when ripe. The variation in the fruit colour is an indication of carotene content. The *nigrescens* has higher carotene content than the *virescens*, while *albescens* has little or no carotene (Purvis 1957; Hartley 1988).

11.3 Oil Palm Breeding

The oil palm industry in Malaysia rooted from the four Bogor palms. Offspring of the Bogor palms planted and selected in Deli, Sumatra formed the basic breeding stock known as the Deli *dura* (Rajanaidu et al. 1979). The first oil palm plantings were therefore of the *dura* fruit form. Despite the narrow genetic base, Deli *dura* fortunately turned out to be a distinctively valuable genotype for breeding and seed production. Fruits of the Deli *dura* are larger and contain a higher proportion of mesocarp, hence a higher percentage of oil compared to their counterparts in Africa (Hardon and Thomas 1968; Hartley 1988). Deli *dura* produces fewer but heavier bunches compared to the African *duras*. Although there are efforts to widen the genetic base of breeding populations worldwide notably in Malaysia, the Deli *dura* is still considered the best *dura* in seed production (Rajanaidu et al. 2000).

An important development took place in The Democartic Republic of Congo (Congo DR), Africa in the 1920s that revolutionised the oil palm industry worldwide. Plant breeders (Beirnaert and Vanderweyen 1941) at the Yangambi Research Station discovered the single gene inheritance for shell thickness and with it the importance of the *tenera* fruit form. Experiments showed that the *tenera* was the hybrid of *dura* × *pisifera* (DxP) containing high mesocarp to fruit ratio, which translates to higher oil yield (Table 11.1). The genetic studies also revealed that the shell gene shows co-dominant monogenic inheritance exploitable in breeding programmes (Beirnaert and Vanderweyen 1941).

Table 11.1 Characteristics of *dura, tenera* and *pisifera* fruit forms

Fruit form Characteristic	*Dura*	*Tenera*	*Pisifera**
Shell thickness (mm)	2–8	0.5–4	Shell-less
Fibre Ring	Absent	Present	Absent
Mesocarp to fruit ratio (%)	35–55	60–96	95
Kernel to fruit ratio (%)	7–20	3–15	
Oil to Bunch (%)	16	26	

* female sterile where bunches seldom develop to maturity.
Source: Hardon et al. 1985; Hartley 1988.

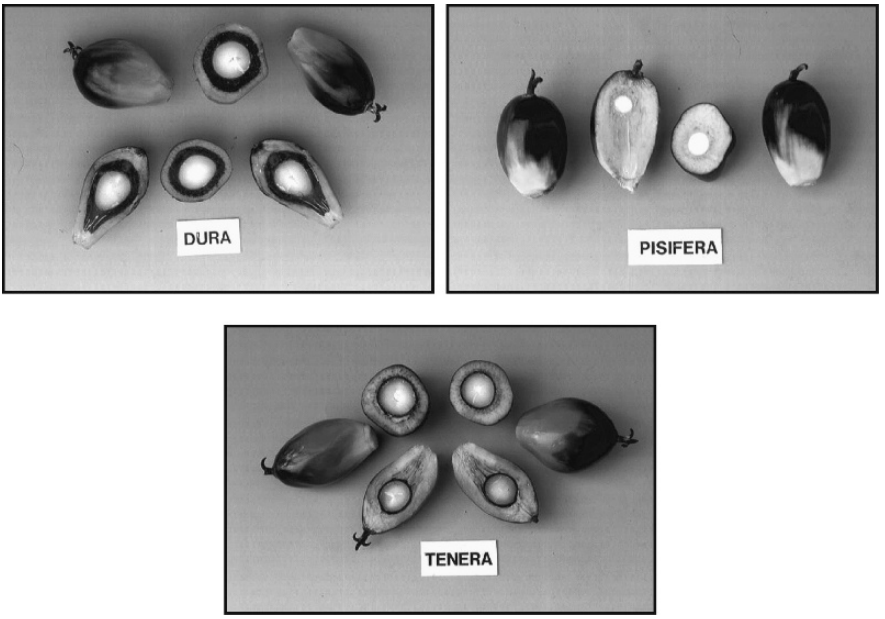


Fig. 11.2 The oil palm fruit (*See* Color Insert)

The higher proportion of oil-bearing mesocarp in *tenera* compared to the *dura* is clearly demonstrated in Fig. 11.2. In this respect, one could consider the *pisifera*, with the absence of a shell and a mesocarp content of 95%, the ideal planting material. However, the *pisiferas* are usually female sterile, i.e bunches usually do not develop beyond the stage of anthesis. The cause of the sterility has not been extensively studied but could be due to the reduced protection of the developing embryo caused by the absence of lignified tissue (Hardon et al. 1985).

Since the crux of oil palm breeding programmes is to produce planting materials with higher oil yield, it was only natural that the findings in Congo DR be exploited. Nevertheless, it was not until 1953, that the Department of Agriculture (DOA) (Malaysia) initiated the first *dura* × *pisifera* plantings in the country (Kushairi et al. 1999a). Initially, *pisifera* pollen was imported from Nigeria and crossed with the Deli *dura* to create D × P progenies whose performance was superior to those of the Deli *dura* progenies. Malaysian oil palm industry quickly responded by switching to the D × P planting materials starting from late 1959 (Kushairi et al. 1999a). Oil palm breeders realised that continuous production of improved D × P planting materials would require the breeding and improvement of parental *dura* and *pisifera* with good combining ability. For this reason, DOA Malaysia embarked on a programme to exchange *pisifera* and *tenera* pollen and seeds with institutes in West Africa under a co-operative breeding programme that was concluded in 1954 (Heath 1955). At the same time DOA collaborated with private plantations in Malaysia to test seed materials from neighbouring Indonesia, which was developing their own breeding programme. One such collaboration between DOA Malaysia and

Harrisons & Crossfield (now Golden Hope Plantations) led to the importation in 1957 of the AVROS[1] *tenera* × *pisifera* (TxP) seeds from Sumatra (Indonesia). The AVROS materials were widely distributed and tested in Malaysia and the resulting AVROS *pisifera* was found to have an excellent general combining ability (GCA) with the *Deli dura* (Kushairi et al. 1999a). The Deli *dura* × AVROS *pisifera* is the most common planting material in Malaysia and worldwide (Kushairi et al. 1999a).

Although considerable success in producing improved planting materials was achieved, leading to the oil palm industry on a solid footing, the narrow genetic pool hampers further improvement. The mother palms in seed production was the Deli *dura* descending from four palms and a limited number of *pisifera* origins as the pollen source (Kushairi and Rajanaidu 2000). This realisation provided the impetus to systematically search for new oil palm genetic materials in its centre of origin, Africa (Hardon 1974; Rajanaidu 1994). The first major expedition by Malaysia to collect *E. guineensis* germplasm was made in Nigeria in 1973. The germplasm was planted in Kluang, a town in southern Malaysia. Subsequently, *E. guineensis* germplasm was also collected in *E.oleifera* collections were prospected in Colombia, Panama, Costa Rica, Honduras, Brazil and Surinam from 1981 to 1982 (Rajanaidu 1986). The subsequent germplasm collections were also planted in Kluang, which now houses the world's largest oil palm germplasm collection. This field gene bank is managed by the Malaysian Palm Oil Board (MPOB). The germplasm are being evaluated and utilised to widen the genetic pool.

Breeding and selection of the germplasm collection to date resulted in the following planting materials and breeding populations:

i. Planting series 1 (PS1): Planting materials with slow height increment (for easy harvesting of fruit bunches) and higher yielding compared to Deli *dura* × AVROS *pisifera* (Kushairi, Rajanaidu, Jalani and Isa 1999b).
ii. Planting Series 2 (PS2): Planting material that produce higher unsaturation palm oil compared to current *Deli dura* × AVROS *pisifera* (Kushairi, Rajanaidu and Jalani 1999c).
iii. Planting Series 3 (PS3): Planting material with a higher kernel content, higher palm kernel oil (PKO) yield. PKO being rich in lauric acid is a feedstock for the oleochemical industry, thus improves productivity (Rajanaidu, Jalani, Kushairi, Rafii and Mohd Din 1996).
iv. Planting Series 4 (PS4): *Elaeis oleifera* planting materials with high carotene content (in excess of 3,000 ppm). Carotenoids are important for human health (Mohd Din et al. 2002).
v. Planting Series 5 (PS5): Breeding population selected for thin shell teneras (Kushairi et al. 2003a).
vi. Planting Series 6 (PS6): Breeding populations selected for large fruit duras. PS6 are intended for the development of mother palms for use in the production of large fruit D × P planting materials targeting for superior absolute oil yield and total economic product (Kushairi et al. 2003b).

[1] *Algemene Vereniging van Rubber Planters ter Ooskust van Sumatra*

vii. Planting Series 7 (PS7): High bunch index breeding populations, which are a way of increasing oil yield (Junaidah et al., 2004).
viii. Planting Series 8 (PS8): Breeding populations that produce oil with high Vitamin E content (Kushairi et al. 2004).
ix. Planting Series 10 (PS10): Breeding populations selected for long stalk, for ease of harvesting (Noh et al. 2005).
x. Planting Series 11 (PS11): High Carotene *E. guineensis* breeding population. The carotene content of this planting series exceeds 2,000 pm, compared to only 500 to 700 ppm for the current D × P material. Planting of PS11 would thus provide additional value to the crop (Mohd Din et al. 2006).
xi. Planting Series 12 (PS12): Breeding population for high oleic acid palm oil, which will be useful for developing planting materials with a more liquid oil (Isa et al. 2006).

The PS germplasm and material were distributed to the members of the oil palm industry in Malaysia for incorporation into their breeding programmes. This provides breeders with new genes for incorporation into the existing breeding stock and further develops the planting materials to produce products for niche markets (example PS3, planting material with a higher kernel content, higher palm kernel oil (PKO) yield for the oleochemical industry).

11.4 Oil Palm Breeding Strategies

Palm oil rose as one of the major vegetable oils traded worldwide and this can be attributed to the expansion of oil palm cultivation in Southeast Asia in the last 40 years. In Malaysia alone, oil palm planted areas grew from 54,000 hectares in 1960 to 4.05 million hectares in 2005. Palm oil production increased in the corresponding period from 94,000 tonnes to 14.96 million tonnes (MPOB 2006). The rapid growth was stimulated in part by progress in research and development (R&D) (Basiron 2000). Discovery of the single gene inheritance for shell thickness and subsequent adoption of D × P planting materials saw a quantum leap in oil to bunch from 16% (*dura*) to 26% (*tenera*). Thus oil palm cultivation became more profitable.

Further yield improvements have subsequently been made through breeding for *duras* and *pisiferas* with good GCA and specific combining ability (SCA) (Yong and Chan 1996). Oil yield as high as 12 t/ha/year has been recorded in an individual palm (Sharma and Tan 1999). This is still below the theoretical yield of about 18 tons (Corley 1998). Breeding research is continuing to make advances especially in the introgression of germplasm with current breeding stocks. This augurs well in reducing the gap between current and theoretical yields.

Oil palm breeding schemes are generally designed to develop both *dura* (D) and *pisifera* (P) populations for the production of commercial D × P seeds. Two breeding schemes may be adopted, namely the reciprocal recurrent selection (RRS) and modified recurrent selection (MRS). The MRS scheme used in Malaysia involves inter-crossing between selected parents. In progeny testing using the North Carolina

Model I (NCMI), a selected *pisifera* parent is crossed with a number of *duras*. If the mean performance of *pisifera* is high with a number of *duras*, then the *pisifera* is regarded to have a good general combining ability (GCA). However, if only selected crosses are high yielding, the *pisifera* is said to have a specific combining ability (SCA). The *pisifera* used in the crossing are usually generated from either *tenera* selfs or crosses (*tenera* × *tenera* [T × T]), *tenera* × *pisifera* (T × P) crosses or *pisifera* × *pisifera* (P × P) selfs or crosses. The *duras* on the other hand are selected from *dura* × *dura* (D × D) selfs or crosses. In the scheme, selected progenies can be carried forward to the next cycle. In this regard the outstanding *dura* palms can be selfed to generate the next cycle of improved *dura* mother palms (Rajanaidu et al. 2000). In the MRS scheme new introductions from other populations can be made to improve the genetic variability. The MRS scheme has proven to be largely successful considering that oil yield has improved from about 0.18 t/ha/yr in the Nigerian grooves to about 3–5 t/ha/yr among Deli *dura* selections (Jalani and Ariffin 1994).

The RRS scheme on the other hand is mainly employed by IRHO and it's affiliates (Rajanaidu et al. 2000). In the RRS scheme, the *dura* and *tenera/pisifera* base populations are kept separate and superior *dura* and *tenera palms* are identified through the performance of D × T and D × P progenies. The parents of the best individual crosses in the progeny testing are selfed and the resulting *dura* and *pisifera* palms are used for seed production. It was reported that the RRS scheme has increased oil yield by almost 18% per cycle compared to the base population (Meunier 1989, Rajanaidu et al. 2000). However, the main disadvantage of the RRS is the requirement of large experimental area to test D × T crosses and selfs.

11.5 Genomics

Genomics is a rapidly developing field that has revolutionised biology, agriculture and medicine. The sciences which are key to genomics research are developing at a rapid rate. The advent of DNA sequencing approaches in the late 1970s and its subsequent automation has had one of the biggest impacts on genome research.

Research in genomics is particularly exciting for higher plants such as oil palm in which the molecular genetics is not as well understood. The oil palm with its long life-cycle and large land requirement for field planting is an ideal candidate for the application of genomic tools for value enhancement. At MPOB, efforts in genomics research are geared towards the generation of diagnostic tools for application in breeding and tissue culture. The generation of Expressed Sequence Tags (ESTs), genetic mapping and application of DNA chip technology are some of the methods employed to enhance the value of oil palm.

11.5.1 Rapid Gene Discovery

In recent years, the dramatic improvement in DNA sequencing technology has resulted in the number of DNA sequences in the world's database increasing

at an unprecedented speed. As of October 2006, *GenBank* (National Centre for Biotechnology Information, URL http://www.ncbi.nlm.nih.gov) contained more than 18.1 million plant entries alone in its dbEST database.

Sequencing of anonymous complementary DNA (cDNAs) from cDNA libraries is a powerful tool for the identification of novel expressed genes and for the comparison of mRNA populations. As few as 150–400 bases are sufficient for searching for a match to sequences resident in the databases (Adams et al. 1991). This approach, frequently referred to as Expressed Sequence Tags (ESTs) has been extensively applied in humans (Adams et al. 1992) and plants (Hofte et al.1993; Lee et al.1998). An EST is usually unique to a particular cDNA and because cDNAs correspond to a particular gene in the genome, ESTs can be used to help identify unknown genes and map their position in the genome. The generation of ESTs has also been used to characterise gene expression in different tissues and stages of development (Fields 1994). The ability to characterise a large number of cDNAs enables a profile of the expressed genes to be created (Weinstock et al. 1994).

The EST approach to gene discovery is especially useful in a plant such as the oil palm in which relatively few genes have been cloned. As of November 2006, there were only 3,092 known sequences deposited at the nucleotide level in dbEST for oil palm in the *GenBank* compared to over 1 million for wheat and maize.

In our effort at rapid gene discovery, cDNA libraries were constructed using mRNA from six different tissues (young etiolated seedlings, mesocarp, kernel, inflorescence, callus and embryoids). To date, over 24,000 cDNA clones have been partially sequenced. Searches made in *GenBank* have so far provided significant matches (score $\geq$ 80) to known genes for about 50% of the sequences. Another 38% of the genes had similarity to known genes but at a very low significance level while the remaining 12% of the sequences did not have any similarity to the genes in public databases. Cluster analysis using StackPack (Miller et al. 1999) software of about 15,000 sequences revealed approximately 9,000 oil palm genes.

Among those clones with significant matches, 90% were identical to plant genes with a majority of them to monocot plants (such as rice, maize and wheat). A diverse group of gene types was identified. The most abundant genes in an inflorescence – specific library were histone and ribosomal protein genes. This library also yielded ESTs for homeobox and zinc finger proteins. From the kernel library, a glutelin and an oleosin gene were identified. Several genes associated with fatty acid biosynthesis were identified from the various libraries screened. They include genes for ß-ketoacyl ACP synthase II (KAS II), acyl carrier protein (ACP), lipid transfer protein and ß-oxacyl-ACP reductase. In callus and embryoid libraries, a large number of genes related to the transcriptional and translation process, such as eukaryotic initiation factor 4A-I5, elongation factor 1-alpha and ribosomal proteins were obtained. This is expected from the active growth state of callus and embryoids used for the cDNA library construction. The genes coding for glutathione-S-transferase (GSTs) were also found to frequently appear in the callus and embryoid libraries. In plants, GSTs are required for normal cellular metabolism and also play a role in cellular response to auxins. The high expression level in the tissue culture material could be due to cellular response to 2,4-dichlorophonoxy acetic acid, used in research as the auxin component of tissue culture media.

About 50% of the ESTs did not have any significant match to any current database entries and therefore represent new, previously uncharacterised genes. A number of approaches can be used to classify these genes, which include expression studies and chromosome mapping. Additionally, the *GenBank* database is constantly growing at a rapid rate. The unidentified cDNAs will also be classified by similarity to genes from other organisms as those sequences become available.

Several of the ESTs identified have potential application in oil palm improvement. The homeobox genes identified are known to be involved in floral development (Ma et al. 1991). As such, they would be ideal candidates for investigation into floral abnormality in oil palm clones. The fatty acid biosynthesis genes such as ACP are useful for in vitro gene manipulation to enhance oil quality in the oil palm. Another interesting clone was the gene for glutelin, which was found in the kernel library. This gene was found to be kernel specific using Northern analysis.

In our strategy for random sequencing, the cDNA libraries were not normalised (normalisation results in all sequences being represented in approximately equal proportions in the library) and the clones were not evaluated prior to sequencing. This resulted in picking up redundant clones sharing the same or nearly the same nucleotide sequence. However in this way, we also obtained clones of gene/protein isoforms, which led to the discovery of gene families. Four groups of ribosomal protein genes have been identified. Several members of the catalase, peroxidase, histone and ubiquitin gene families were shown to be represented in the libraries analysed. Northern blot analyses have shown that different members of a gene family could exhibit differences in tissue specificity. In addition, differences in the nucleotide sequence have already proved useful for restriction fragment length polymorphism (RFLP) mapping of each gene within the gene family (Sasaki et al. 1994).

The value of ESTs has been widely acknowledged. In oil crops, some of the oil synthesizing enzymes are membrane bound. As such, the conventional methods of cloning through biochemistry are very difficult for the genes of these enzymes. However, Sommerville and co-workers were able to obtain these genes serendipitiously among ESTs generated from *Arabidopsis thaliana* (Glaser 1996). These were later used to search for similar genes in the economically important oil crops. The EST approach to gene discovery should therefore also prove useful for oil palm. Furthermore, ESTs are also an excellent source of probes for DNA microarray analysis, an experimental technique which allows the analysis of the expression patterns of thousands of genes in parallel. ESTs are also incorporated into the ongoing genome mapping programme at MPOB. They have proven to be an excellent source for probes of known identity and as such the molecular maps generated are of known genes. The use of known ESTs, especially those related to tissue specificity and developmental phases, can eventually lead to a better understanding of how genome organisation affects gene expression during development.

Ideally, ESTs generated from various cDNA libraries should represent all or a very large percentage of expressed genes in an organism. However, the expression profiles of different genes in different tissues can give rise to mRNAs that differ in abundance, making it difficult to capture rare mRNAs from cDNA libraries (Reddy et al. 2002). Rapid gene discovery techniques also lead to redundant sequencing

where the same gene cloned can be sequenced several times. This is a problem in large-scale sequencing projects and has also been observed in our programme of rapid gene discovery in oil palm. To overcome the redundancy problem and capture more genes, especially the rare transcripts, efforts were also directed towards constructing a normalised library. The existing polyembryoid cDNA library was normalised using a modification of the reassociation kinetics method described by Bonaldo et al. (1996). The normalised library was successfully constructed with about 10,000 gene clones. A total of about 1,000 clones have been sequenced to date from the normalised library and analysis largely reveals that the normalisation process has been successfully carried out. The normalisation process was confirmed by dot blot hybridisation and sequencing, whereby redundancy of abundant genes like ribosomal L2 (50S) protein, metallothionein-like-proteins and lipid transfer proteins was reduced by more than 20, 2 and 7 fold respectively. At the same time the sequencing data revealed that normalisation increased the frequency of lower abundant genes captured such as pectinerase by about 12 fold and early nodulin genes by about 14 fold. The frequency of genes observed in the normalised and non-normalised library is summarised in Table 11.2. A number of genes not sequenced previously in the cDNA library were captured in the normalised library. Examples of these include auxin binding protein, diphenol oxidase and glycosyl transferase family 8 protein. The normalised library is currently being further sequenced and we are confident of increasing the number of genes captured for oil palm.

The increasing number of sequences being generated at MPOB has led to numerous problems such as efficient data management and analysis. At the same time it has also provided unlimited opportunities to further unravel the molecular

Table 11.2 Comparison of frequencies of known genes in EO library vs EON library

	Percentage (Frequency)*		
	EO library[a]	EON library[a]	EON library[b]
Lipid transfer protein	0.780 (27/3459)	0.099 (1/1010)	0.099 (1/1002)
Cys-peroxiredoxin	0.491 (17/3459)	0.000 (0/1010)	0.000 (0/1002)
Pectinesterase	0.058 (2/3459)	0.693 (7/1010)	0.699 (7/1002)
Ribosomal L23A (60S)	0.115 (4/3459)	1.386 (14/1010)	0.898 (9/1002)
Metallothionein-like protein	0.838 (29/3459)	0.396 (4/1010)	
Ribosomal L2 (50S)	0.318 (11/3459)	0.000 (0/1010)	
Lectin	0.318 (11/3459)	0.099 (1/1010)	
PVR3- like protein	0.347 (12/3459)	0.198 (2/1010)	
PBS lyase HEAT-like repeat-containing protein	0.000 (0/3459)	0.500 (5/1010)	
Pyruvate dehydrogenase kinase isoform I	0.000 (0/3459)	0.594 (6/1010)	
Early nodulin	0.029 (1/3459)	0.396 (4/1010)	

[a]Data based on sequencing analysis;
[b]Data based on dot blot analysis.
*Frequency = No. of positive clones identified/total no. of clones analysed;
*Precentage (%) = frequency × 100.
EO: Conventional polyembryoid cDNA library.
EON: Normalised polyembryoid cDNA library.

build-up of this important plantation crop. To harness this information effectively, an automated sequence analysis and data management system is essential. In response to this, MPOB in partnership with a private bioinformatics company established an automated bioinformatics pipeline and database known as *PalmDNABase*. *PalmDNABase* is an integrated DNA sequence management system that captures data automatically from the sequencer. The graphical interface of *PalmDNABase* is based on HTML and JAVA and runs on a LINUX operating system. The system has in place tools for data analysis that allow systematic storage of sequence information and analysed data. The processing workflow makes use of tools such as PHRED, VecMask, LUCY, CAP3 and BLAST that allow validation of sequence quality, vector masking and trimming, clustering and homology search. The sequences are automatically annotated according to the BLAST results and can be automatically prepared for submission to dBEST or *GenBank* at the National Centre for Biotechnology Information (NCBI). The establishment of *PalmDNABase* system has proven extremely helpful in managing and analyzing the sequence data available at MPOB.

Information on these gene clones sequenced at MPOB is also being deposited in *PalmGenes*, which is the first oil palm DNA database to be publicly available on the internet. *PalmGenes* was developed at MPOB and can be accessed at (http://palmoilis.mpob.gov.my/palmgenes.html). *PalmGenes* serves as a knowledge bank and facilitates querying of genes expressed in oil palm. The genes in this database have also been characterised into 18 functional categories for ease of reference.

The information available on *PalmGenes* is as follows:

- Sequence data of gene clone.
- Similarity to known genes.
- Cloning vector used and gene insert size.
- Functional characterisation.

The database currently provides relevant information on about 6,000 gene clones. *PalmGenes* is a dynamic database, which will evolve and grow with time.

11.6 Molecular Breeding

Molecular markers and genetic mapping are part of the intrusive ‘new genetics’ that is thrusting its way into all areas of modern biology (Jones et al. 1997). The location of markers and the relative distances from one another along each chromosome of an organism represents a genetic map. Genetic mapping provides a means of determining the location(s) of genes influencing a specific trait of interest and, as such, the information needed to implement marker assisted selection (MAS). The tagging of markers closely linked to loci governing agricultural productivity are useful selection tools in plant breeding. This concept, which has also been dubbed as ‘molecular breeding’ (Rafalski and Tingey 1993), is conventional breeding aided

by DNA-based diagnostics. The main advantage of molecular breeding is that plants can be selected early, even before the characteristic in question is manifested. This greatly reduces the time required for bringing new varieties to the market (Mazur and Tingey 1995). It is also claimed that selection using molecular markers is more precise.

Molecular breeding is well suited to a perennial crop like oil palm in which the economic products are not produced until several years after planting. The oil palm has a long selection cycle of 10–12 years (Mayes et al. 1997). As such, achieving genetic gains through conventional breeding can be an extremely slow and tedious process. This fact is further confounded by the fact that oil palm requires large tracts of land for field planting as only about 140–160 palms are planted per hectare. The large land requirement directly translates to huge manpower requirement to manage and conduct breeding trials. This result in breeding trials are often becoming very expensive and limiting in terms of the number of crosses that can be evaluated. The use of DNA markers for selection, in a crop like oil palm, could greatly reduce the number of breeding cycles. The use of DNA markers, especially by assessing their allelic variation at agriculturally important loci, can assist in making informed choices on the palms to be selected for crossing and/or subsequent field planting. These are decisions that will help to efficiently utilise the limited resources currently available in Malaysia with regards to land and labour shortage in conducting breeding trials. As such, planting materials can be produced faster and with greater precision. It is also argued that molecular breeding can assist the oil palm achieve its real potential oil yield, which is projected to be about 18 tons oil per hectare per year (Corley 1983, 1998; Uoti and Jacob 2001). In Malaysia, the national average yield in 2005 was about 3.8 tons oil per hectare (MPOB 2006), almost five-fold below its estimated potential.

Projects have been designed at MPOB to develop tools and techniques for molecular breeding in oil palm (Cheah et al. 1999). The first phase of the project was targeted towards the development of molecular markers. Since there was already an 'in-house' collection of ESTs, it was only natural that they be exploited as restriction fragment length polymorphism (RFLP) markers. RFLP markers are co-dominant, robust and easily transferable among different laboratories. The use of ESTs to reveal RFLP offers an added advantage that a map of known genes can be obtained. In the oil palm, the two single gene inherited traits (monogenic inheritance) of importance to plant breeders are shell thickness and fruit colour. A marker linked to the shell thickness character is of significant importance as it would allow breeders to distinguish the *dura, tenera* and *pisifera* types at the nursery stage. It would also allow breeders to eliminate dura contamination from the commercial *tenera* planting materials. As for fruit colour, the *virescens* (*Vir*) fruits undergo a more profound colour change on ripening and as such makes it easier for harvesters to identify ripened bunches. The identification of the *Vir* allele and its use in conjunction with the non-abscising genotypes will allow the identification of ripened bunches and hence reduce crop loss through fallen fruits (Jack et al. 1998).

A progeny derived from the selfing of a tenera palm (Palm T128 from MPOB's Nigerian germplasm collection) and segregating these two monogenic characters

was used for linkage map construction. Initially RFLP markers were used in the mapping effort. cDNAs derived from the young etiolated seedlings, mesocarp, kernel and inflorescence were used as probes. Since the oil palm is an outbreeding species and a high degree of heterozygosity is expected in its genome, the co-dominant RFLP markers are expected to segregate in a 1:2:1 ratio in this selfed cross. An initial attempt at map construction using RFLP markers detected 11 linkage groups (Cheah et al. 1999). The RFLP technique, however, suffers from the disadvantage of being labour-intensive and time consuming. Thus the AFLP technique (Vos et al. 1995) is also being applied to the oil palm in an attempt to expedite the mapping programme (Rajinder et al. 1998). AFLP markers were scored as dominant and are expected to segregate in a 1:3 ratio in the selfed progeny. Work is also being carried out to develop markers based on simple sequence repetitive (SSR) DNA, or micro-satellites. These markers are also co-dominant and potentially useful for map saturation. Together with RFLP markers, SSR can serve as anchor probes for the development of a consensus map.

11.6.1 Linkages Between Genetic Markers and Genes Influencing Monogenic Traits

A total of 123 RFLP markers, 69 AFLP primer pairs and 23 SSR markers were used to screen 136 progeny palms of the T128 selfing. The linkage map was constructed using JoinMap (Version 3) (Van Ooijen and Voorips 2001). The current map consists of 17 linkage groups with 117 RFLP loci, 384 AFLP markers and 23 SSR markers over 1,677 cM (Rajinder 2005). More than 90% of the segregating markers identified could be mapped, suggesting good genome coverage. Currently we are attempting to identify additional SSR markers in order to resolve the number of linkage groups to the basic haploid chromosome set of 16.

In the current linkage map, we have successfully tagged the fruit colour gene (v*ir*) on Group 3. Two interesting linkages were identified to the fruit colour gene, MET16 (3 cM) and KT3 (4 cM). Both MET16 and KT3 are RFLP probes that showed co-dominant profiles. Figure 11.3a and b illustrates the linkage of both probes to the *Vir* gene by displaying a sub-set of the mapping population analysed with these probes. A majority of the *Vir* fruit (93%) matched homozygous (top segregating band present) or heterozygous (both the segregating bands present) profile of the probes. At the same time, a very large proportion of the *nigrescens* fruits showed a profile consistent with only the bottom segregating band being present (homozygous for the alternative allele). Both probes show promise in not only being able to distinguish the *nigrescens* and *Vir* fruits but also the ability to distinguish the homozygous and heterozygous forms of the *Vir* trait. These markers are most useful as they will allow for a breeding strategy that only uses palms that are homozygous dominant for the Vir^{+} alleles in their crossings. This will ensure the presence of only the *Vir* type in the progeny and eventually lead to easy identification of ripe bunches.

The field data for shell thickness is also being examined in order to identify the locus controlling this character. However, markers associated with the shell

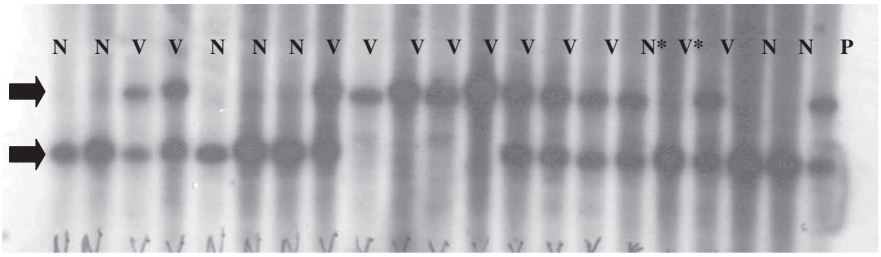


Fig. 11.3a Segregation of the marker MET16 compared with that of the fruit colour trait in the mapping population. N is nigrescens and V is virescens fruits. "*" indicates fruit colour not matching the banding profile, P = parental palm

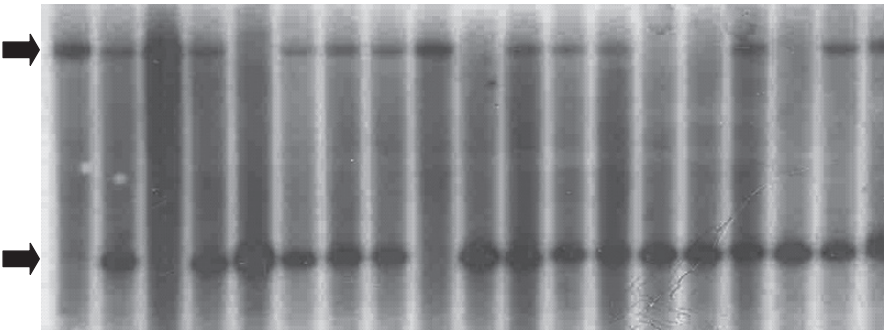


Fig. 11.3b Segregation of the marker KT3 compared with that of the fruit colour trait in the mapping population. N is nigrescens and V is virescens fruits. "*" indicates fruit colour not matching the banding profile, P = parental palm (*See* Color Insert)

character of the fruit have been identified using RFLP (Mayes et al. 1997), RAPD (Moretzsohn et al. 2000) and AFLP (Billotte et al. 2005) marker systems. The closest marker linked to the shell thickness loci is an AFLP marker, which was mapped about 5 cM away from the shell thickness loci (Billotte et al. 2005). The marker is still too far way to allow for an error free selection of the trait in the nursery.

11.6.2 Linkages Between Genetic Markers and/or Genes Influencing Quantitative Traits

With the availability of genetic linkage maps, another important step towards genetic analysis of agricultural productivity is the analysis of quantitative inheritance (Paterson et al. 1991). For monogenic traits, a single gene virtually explains 100% of the variation observed for the character. However, most of the measures of agricultural productivity, such as yield and quality, are under polygenic control (controlled by many genes). These polygenes or QTLs (Geldermann 1975) can be identified as in monogenic traits with linkages to genetic markers (Paterson

et al. 1991). As such, the location of individual QTLs can be determined via molecular mapping.

In our attempt at mapping QTLs associated with oil quality in oil palm, we examined an inter-specific cross (Colombian oleifera [UP1026] crossed with Nigerian guineensis [T128]). The parameters analysed were iodine value (IV) and fatty acid composition (FAC). IV is a measure of the unsaturation of fats and oils. In order to allow for both edible and non-food usability of the oil, increasing the proportion of unsaturated fatty acid (particularly oleic acid) is desirable. As such, QTLs for IV and FAC will allow for early selection of desirable palms.

In order to map QTLs associated with oil quality, linkage analysis was first carried out for the inter-specific cross. The inter-specific cross was mapped using the pseudo-testcross strategy described by Grattapaglia and Sederoff (1994). In this method, each parent is in turn considered heterozygote while the other homozygous. Markers are thus expected to segregate in a 1:1 ratio. Again RFLP, AFLP and SSR markers were used. In the mapping of this cross, it was found that the majority of the markers (about 80%) was contributed by the male parent, *E. guineensis*. It therefore appears that the guineensis genome has much higher heterozygosity than the oleifera. The effort to map this cross as a one-way pseudo-testcross with guineensis male parent as the heterozygote scored 418 AFLP, 74 RFLP and 22 SSR segregating markers in a mapping population of 117 palms. In total, 380 markers (295 AFLP, 65 RFLP and 20 SSR) were mapped in 18 linkage groups (Rajinder 2005). The total genetic distance covered by the markers was 1,571 cM, with an average interval of 4.1 cM between adjacent markers. About 85% of the markers could be linked to at least one other marker indicating good genome coverage.

QTL analysis was performed for each of the measured traits using the interval mapping method implemented by MapQTL version 4.0 (Van Ooijen 2002). At a genomic wide significant threshold of $P < 0.01$ and $P < 0.05$, significant QTLs were detected for IV, C14:0, C16:0, C16:1, C18:0 and C18:1 (Table 11.3). The QTLs for C16:0 and C18:1 were mapped on the same group (Table 11.3). All three QTLs showed similar shaped likelihood profiles (data not shown) suggesting that the same QTL may be influencing the three traits. The fact that the traits are significantly correlated further supports this assumption. C18:1 is the most abundant unsaturated fatty acid while C16:0 is the most abundant saturated fatty acid in palm oil. As such, it is of no surprise that the same locus could be influencing the three traits. QTLs were also detected at the $P < 0.01$ significance threshold levels for C14:0 (in Groups 4 and 12), C16:1 (Group 12) and C18:0 (Groups 3 and 12). Significant QTLs for C16:1 and C18:0 were detected around the same region in Group 12. This probably points to another major locus influencing fatty acid composition in the oil palm.

Apart from Interval Mapping, the rank sum test of Kruskal-Wallis was also used to detect markers with linkages to specific QTLs. This was to establish if there was an agreement between both tests (Interval Mapping and Kruskal–Wallis). The Kruskal–Wallis test is regarded as the non-parametric equivalent to the one-way analysis of variance (Van Ooijen and Maliepaard 1996), and was also carried out using the MapQTL software. The results are also summarised in Table 11.3. Generally the results from both analyses were in agreement. For all traits, the markers

Table 11.3 QTLs for IV and fatty acid composition found to be significant at the empirical genome wide mapping threshold

Trait	Genome wide significant level $P < 0.05$	Genome wide threshold $P < 0.01$	Group	Closest Markers	Position (cM)	LOD Score	Kruskal–Wallis test	% variance explained
IV	3.8	4.7	1	CB75A	40	7.75	P = 0.0001 (K = 26.19)	35.6
C14:0	3.5	4.7	4	TAAC/HC AA-130	65	4.07	P = 0.0005 (K = 13.92)	20.9
			12	P4T8	22	4.25	P = 0.0001 (K = 18.59)	21.7
C16:0	4.6	5.7	1	CB75A	40	7.98	P = 0.0001 (K = 27.72)	36.8
C16:1	3.7	5.0	12	P4T8	22	10.53	P = 0.0001 (K = 34.98)	45.5
C18:0	3.3	4.0	3	EACA/MC AG-219	40	3.59	P = 0.0005 (K = 12.57)	18.9
			12	P4T8	22	4.0	P = 0.0001 (K = 16.22)	20.6
C18:1	4.0	4.8	1	CB75A	40	5.71	P = 0.0001 (K = 23.5)	28.3

with the largest LOD score on the linkage group (which is the estimated position of the QTL) also showed very high significance for the presence of a segregating QTL in the Kruskal–Wallis test. The two tests appear to point to a common locus influencing QTLs associated with fatty acid composition.

In this study eight (8) QTLs were detected for IV and the five components of fatty acid composition (C14:0. C16:0, C16:1, C18:0 and C18:1) in four different linkage groups. Since similar work has not been carried out for oil palm prior to this, a direct comparison with findings from other research groups could not be made. However, a comparison with other crops (mainly annual crops) is possible. For example, in maize, Alrefai et al. (1995) detected 15 QTLs (in 8 groups) associated with C16:0 only. Similarly, Mangolin et al. (2004) detected 13 QTLs distributed in 8 chromosomes for kernel oil content in maize. The low number of QTLs detected in this study was, however, in agreement with the work done by Somers et al. (1998) and Jourdren et al. (1996). They found that a few QTL loci could explain a big proportion of the phenotypic variation associated with one of the fatty acid components, C18:3 (linolenic acid) in *Brassica napus*. Nevertheless, it is important to note that the differences in QTLs mapped in this research cannot be directly compared to those reported above because of the different crop, type of markers, mapping population structure and the density of the genetic maps used in the analysis. Currently, efforts are also ongoing to map loci affecting tocopherol/tocotrienol contents in oil palm.

The effectiveness of markers and genetic maps continues to increase and, aided by the development of high throughput assay systems, molecular breeding is not only attractive but is also becoming more affordable. Furthermore, since variety improvement through molecular breeding does not involve transformation or integration of alien genes into the genome, it is not complicated by the issues of biosafety and bioethics.

11.7 Genome Analysis Through DNA Microarray

As a consequence of the need to simultaneously monitor the expression levels of large numbers of genes, several valuable technologies have been developed. One of the most robust and exciting technologies developed in modern biology has been the DNA microarray or the so-called DNA chip. The array technique pioneered by Pat Brown and colleagues (Lander 1999) has found widespread application in gene discovery, disease diagnosis and drug discovery. The massive capability of the DNA microarray also lends itself to several important applications in plants. An understanding of complex developmental processes, such as vegetative growth, flowering and fruit formation, can be greatly enhanced using this technique. Traits that contribute directly to the economic products of a crop, for example, nutrient uptake, response to fertilisers, ability to withstand stress and plant pathogen interactions, are also amenable to analysis by this technique.

In the oil palm, the availability of a large number of ESTs provides the platform for large-scale functional analysis of the genes using microarray. One aspect of the

functional genome analysis is to understand the temporal and spatial expression patterns of the genes in specific tissues. At MPOB, sequences generated from ESTs and the in-house collection of oil palm genes have been printed on glass slides for research investigations using the DNA microarray technology. To date, about 3,600 oil palm gene clones have been printed. Preliminary investigations were carried out to establish the technique and determine the feasibility of this technology for gene expression profiling in the oil palm. The expression profiles of different tissues were compared to determine differential gene expression and identify tissue specific expression.

One of the first experiments carried out was to compare the expression profile between leaf and callus tissues. Among genes found to be up-regulated in callus were aquaporin, ribosomal proteins and histones. Since the callus is an actively dividing tissue, the up-regulation of genes involved in transcription and translation such as ribosomal proteins and histones is expected. Aquaporin facilitates the transport of water and small neutral solutes across plant cell membranes. They are especially critical in the early growth and enlargement of plant cells. For this reason, the up-regulation of aquaporin in callus cultures is desirable for maintaining the physiological function of the cultures. Genes involved in stress response and detoxification, such as glutathione S-transferase and metallothionein-like protein, were also highly expressed in callus samples. The over-expression of stress genes in callus tissues is to be expected, considering that this is the first development stage of oil palm tissue culture and that the tissues are adjusting to a new environment. From this preliminary microarray analysis, the expression patterns of many genes on the chip could be identified. The possible role and function of the genes in specific tissues could be ascertained from their expression profile and published reports of the gene function in other species.

The microarray technology is very valuable in understanding the complexity of gene expression in oil palm tissue culture. In oil palm tissue culture, the recovery of viable plants is not efficient and not routinely implemented on a large scale at present. The success of tissue culture is dependent on genotypes used for culture and the process also suffers from a low rate of embryogenesis. Analysing the expression pattern of genes during the different development stages of tissue culture could provide an insight into the fundamental mechanisms that underlie oil palm tissue culture. Our primary focus in the tissue culture experiments was to identify oil palm genes differentially regulated in the developmental process. Materials for analysis were sampled from explants at various stages during the tissue culture process.

A number of genes were found to be expressed after 7 days in culture (e.g. heat shock protein, pathogenesis-related protein and MAP kinase 5). The heat shock proteins and pathogenesis-related proteins are stress related genes, while MAP kinase 5 is associated with stress and senescence. The up-regulation of stress genes is expected since the explant is adjusting to it's new environment in the culture media. The up-regulation of the senescence gene is also logical considering the leaf explant used is separated from the mature palm and is in an artificial environment. An early flowering protein was also found to be expressed in culture (after seven days of culture), with its expression maintained up to two weeks in culture. This would indicate

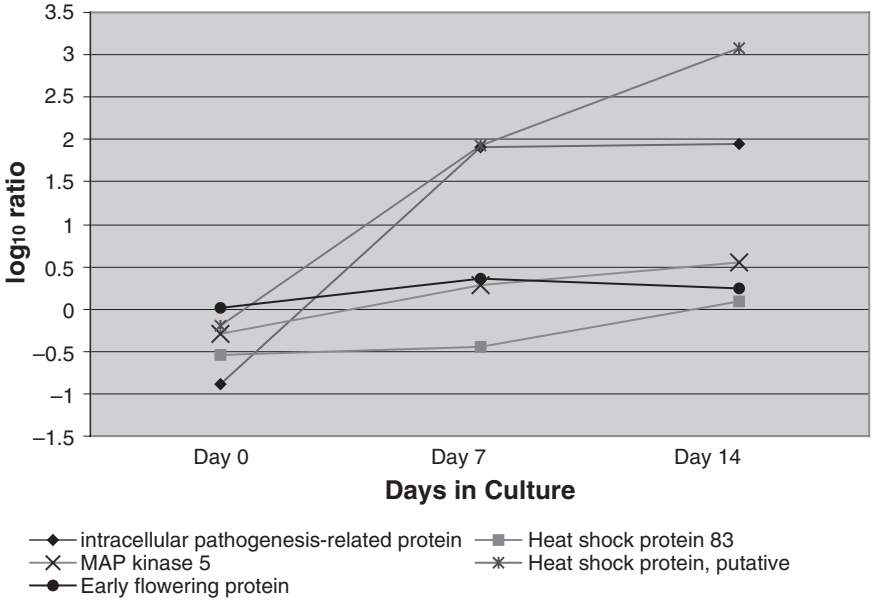


Fig. 11.4 Examples of genes regulated during oil palm tissue culture (*See* Color Insert)

that genes associated with reproductive organs are also expressed early in oil palm tissue culture. The expression levels of the selected genes during the tissue culture process is shown in Fig. 11.4. Generally, a large number of genes show a change in expression profile as the samples develop in culture, indicating that a wide range of genes is being regulated at least in the early stages of the culture development.

11.8 Current Trends in Tissue Culture

The approach of enhancing competitiveness by the expansion of land for oil palm to raise production is no longer a viable option. The only viable alternative is to increase productivity per unit land area, i.e. increase yield and profitability per hectare on the same land. Attaining superior oil yield per unit area of the oil palm is the most important avenue for future competitiveness and sustainability of the oil palm (Simon and Koh 2005). The current method of propagating high yielding materials is through cross pollination of selected seed materials with known improved genetic potential. Although rigorous selection is carried out, the problem of segregation in the seed derived progenies still persists. The other hitch to take heed of is that the current achievement of an average oil yield of 3.8 tons per hectare per year (MPOB 2005) is way below the reported genetic potential of the oil palm of 18.5 tonnes per hectare per year (Chan 2000). At this juncture, clonally propagated oil palm is the best possible solution to realise this hidden potential. However, it should also be noted that for oil palm, tissue culture is not an independent discipline.

In order to ensure a successful venture in the production of clonal oil palm materials, a good established breeding programme is essential for the identification of superior mother palms or ortets.

Over the years, the in vitro propagation process of the oil palm has stayed much the same (Rohani et al. 2000). Many published data and reviews have quoted the importance and viability of tissue culture of the oil palm and have also described the pitfalls of the technique (Jones 1974; Rabéchault and Martin 1976; Khaw and Ng 1997; Rohani et al. 2000). However, over time, researchers in this area have gained tremendous experience and this has enabled them to fine tune the propagation process. With increased confidence backed by data collected over the years on the fidelity of clonal materials (Soh et al. 2001; Maheran and Abu Zarin 1999; Ho 2000; Zamzuri et al. 2005), Malaysia is now keen to promote the use of clonally propagated oil palms as a source of high quality material for replanting nationwide.

11.8.1 Fast Tracking Breeding Programmes via Tissue Culture

In relation to this, MPOB having the largest oil palm germplasm collection in the world has continuously evaluated its collection for traits of economic interest and value (Mohd Din et al. 2005). Acknowledging the fact that producing new and improved planting materials through conventional breeding is time consuming, tissue culture has now become an appropriate strategy adopted for expediting the development of commercial planting materials.

One of the setbacks in the tissue culture programme is the high number of ortets required to meet the production demands of the laboratories. Ortets are usually selected from D × P and D × T progeny trials where performance data are available. Ortets can also be selected from T × T or T × P crosses usually carried out in the *pisifera* improvement programme (Yong and Mustafa Kamal 2005). Alternatively, to add to the pool for selection of ortet palms, Soh et al. (2001) suggested cloning from seedlings of elite D × P due to the low heritability of yield between palms. There are also cases where selection was done from highest yielding commercial fields (Yong et al. 2005). One of the latest developments in overcoming the limitation due to insufficient supply of parental materials is the introduction of semi/bi-clonal seeds and clones (Yong and Mustafa Kamal 2005; Sharma 2006). Semi-clonal seeds are produced through the crossing of *dura* clones with normal *pisiferas*, while bi-clonals are when both *dura* and *pisifera* parents are cloned and multiplied then utilised as parents for seed production (Mohd Din et al. 2005). Several advantages of semi-clonal and bi-clonal seeds over the current D × P seeds have been highlighted. The semi/bi-clonal seeds apparently have greater degree of uniformity as they are derived from a limited number of parental genotype combinations. Their cost per seed is much lower than that of tissue cultured plants and they have a lower risk of mantled fruit abnormality. It is also less demanding infrastructure-wise as the number of parents required to be cloned and the number

of ramets required per clone is small. Ultimately, an oil yield gain of between 15 and 25% compared to conventional mixed batch of D × P hybrid seeds can be expected (Sharma 2006).

11.8.2 Innovations for Improvement in the Tissue Culture of Oil Palm

The tissue culture process has then and again been shown to be an extremely valuable alternative to conventional breeding. However, it is also known to be a costly and labour-intensive substitute. The whole process requires very specialised infrastructure to ensure a clean and controlled environment, ample laboratory space to house the cultures and, most importantly, skilled workers, the lifeline of any tissue culture laboratory. Therefore, any cost and labour reduction strategies introduced would be very much welcomed. Since the new millennium, MPOB has been actively developing innovations that can help improve the efficiency of the tissue culture process.

In oil palm tissue culture, shoots of an appropriate age are individually transferred into a fresh medium for rooting or elongation purposes (Zamzuri et al.1998; Zamzuri 2001). This labour-intensive exercise is now being replaced by the "double-layer rooting" technique. This technique involves pouring or adding liquid medium directly onto the established cultures instead of transplanting it individually into a new medium. This innovation has reduced the handling of individual shoots and has increased the working capacity by as much as 17.5 fold at the rooting stage. The culture room area can be reduced by 2.6 fold if bigger culture vessels are used instead of the test tubes. Annual variable cost on wages and culture medium was reduced by as much as 80%.

An award winning invention that has also found a niche in the laboratories is the "flameless steriliser" (Zamzuri 2002). This electrical device was designed to replace the conventional heat sterilisation procedure via flaming of tissue culture glass containers and surgical apparatus. The flameless steriliser has a hot coil that is able to disperse heat as high as 300 °C and allows dual surface sterilisation at the same time (mouth parts of the vessel and surgical apparatus) thus maximising the use of the heat generated (Fig. 11.5). This form of sterilisation is free of flame, fumes and is hands-free while vessels are being sterilised.

Several laboratories, in addition to MPOB, have invested in developing liquid culture system as it promises reproducibility, versatility and efficiency with the prospects for automation (de Touchet et al. 1991; Teixeira et al. 1995; Wong et al. 1999; Tarmizi 2002). Although the shake flask system is sufficient to cater for the needs of some laboratories, it may not be enough if rapid large-scale proliferation is required. In this case the bioreactor is the preferred alternative (Tarmizi and Zaiton 2005). Bioreactors have been proven to successfully multiply suspension cultures of several plant species (Okamoto et al. 1996; Yu et al. 2000). In relation to this, the MPOB Fast Transfer Technique (MoFaTT) in liquid culture system (Tarmizi and

Fig. 11.5 The flameless steriliser (Source: Zamzuri 2002)

Zaiton 2005) was developed (Fig. 11.6). This system allows a convenient and rapid replenishment of liquid media on site with reduced risk of contamination. Other improvements to the system such as the 2-in-1 MoSlim (MPOB simple impeller for liquid culture; Fig. 11.7) which is a modified impeller used for agitation, and the combination of the two systems forming the "simple impeller with fast transfer technique" for liquid culture system (SLIM-FaTT) was recently developed to further enhance the liquid culture process (Tarmizi and Zaiton 2006a,b).

During the early days of tissue culture, data entries were manually done and their records kept in files. With the advent of the information technology these data were digitised and archived in hard discs for future use. However, with the current scenario merely digitising the information is no longer sufficient. There is now a need for proper management of the flow of information generated from the laboratory right up to the field. In addition, there is also a need to integrate the information with that gathered from other disciplines such as breeding and molecular biology. The Oil Palm Tissue Culture Tracking System (OPTRACKS) was developed to perform tracking of the tissue culture process. The system stores detailed information of each step of the tissue culture process starting with ortet introduction, callus formation and multiplication, embryogenesis, embryoid multiplication, shoot development and rooting of plantlets, hardening and conditioning in the nursery. This information can be easily obtained by scanning the bar code label containing

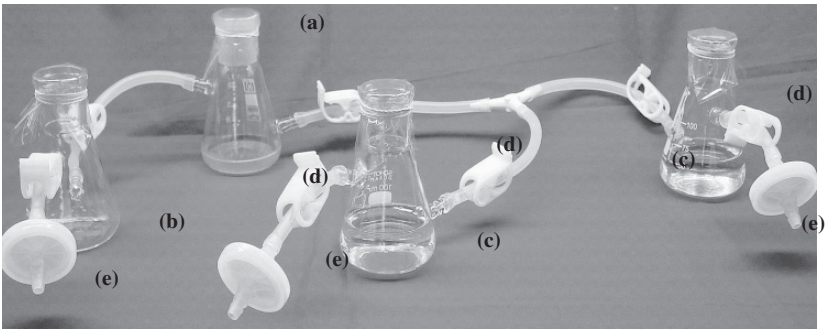
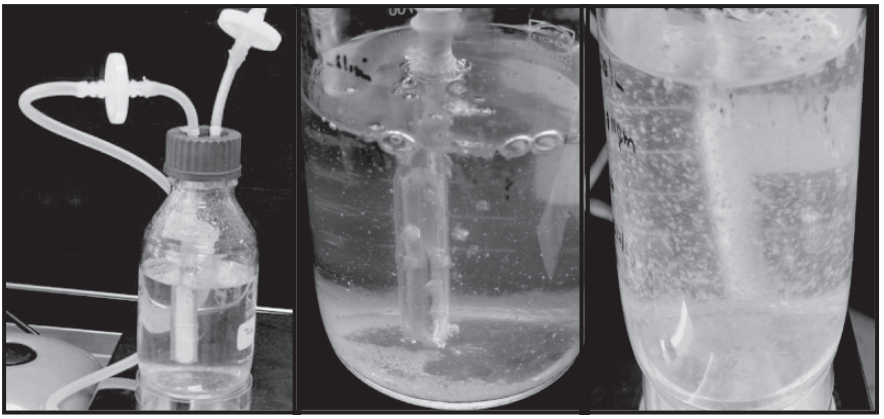


Fig. 11.6 MPOB fast transfer technique (MoFATT) in liquid culture system that allows easy monthly medium replenishment. (**a**) Pre-sterilised flask of any size with media for culture maintenance. (**b**) Empty pre-sterilised flask of any size for discarding spent medium. (**c**) Pre-sterilised flasks of any size with fresh maintenance, maturation or other specific media. (**d**) Clamps are attached to the tubing and are released during medium replenishment. (**e**) Filter devices are attached to each flask to reduce the pressure build-up within the flask
(Source: Tarmizi et al. 2005)



(a) (b) (c)

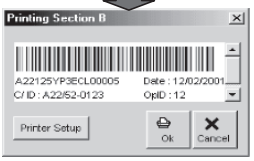
Fig. 11.7 The 2-in-1 MoSlim (MPOB simple impeller for liquid culture). (**a**) An example of the set-up with a modified vessel with the simple impeller. (**b**) Closer view of the simple and economical impeller. (**c**) The 2-in-1 system allows simultaneous agitation and aeration
(Source: Tarmizi and Zaiton 2006a)

the production batch and sequence number which is then tagged onto the culture vessels used and the nursery plant (Fig. 11.8).

DNA fingerprinting has also become one of the routine techniques incorporated in tissue culture and breeding. From the previous application of restriction fragment length polymorphism technique for DNA testing (Cheah et al. 2002), it has now advanced to using micro-satellite or simple sequence repeats (SSR) probes (Rajinder et al. 2006). These probes are basically used as a means of quality control in the oil palm tissue culture process. Through this technique the identity of the clones can

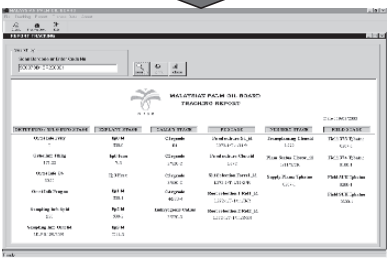
The OPTRACKS screen appears for Users to enter the system

Entering relevant data for particular stage of cultures



A bar code label is produced, printed and used to tag petri dishes, jars, flasks, nursery or field palms

Tracking by using scanner on bar code labels



This system will display the detailed information for particular stage of cultures

Fig. 11.8 Process flow in OPTRACKS by using the bar coding system
(source: Tarmizi et al. 2005)

be determined, thus allowing the detection of possible culture mix-up. This can also be extended to verification of the authenticity of the clones during a recloning exercise. It is known that plantlets derived from in vitro culture techniques might exhibit somaclonal variation that is often heritable (Larkin and Scowcroft 1981; Breiman et al. 1987). Therefore, it is important that clones are constantly monitored throughout the tissue culture process to ensure that there is uniformity between and within lines.

11.9 Biomarkers in Tissue Culture

Tissue culture's most prevalent issues are the low embryogenesis rate and clonal abnormality. MPOB has been actively involved in gene expression studies in both embryogenesis and clonal abnormality mainly with the aim of discovering and to develop possible biomarkers to address the issues of tissue culture amenity and abnormality.

To date, MPOB has been able to determine some possible markers for embryogenesis (Fig. 11.9). Based on the expression studies conducted, EgPER1, EgHOX1, EgPK1 and OPSC10 could be targeted at early somatic embryogenesis when callus cultures are still on solid media. Whether these proteins are involved during the induction of embryogenesis remains unknown. Functional studies have to be carried out to elucidate the functions of these proteins during embryogenesis. OPHb1 most probably plays a part at a later stage during meristem establishment. As for the other genes, e.g. RLKs, Calmodulin (OPZE3) and QM (OPZE5) proteins, as their expressions are more ubiquitous they could be postulated to be involved in a more general mechanism during somatic embryogenesis (Ong-Abdullah and Ooi, submitted).

Clonal abnormality issue was once considered the major cause for crippling the progress of commercially producing clonal oil palm elite materials. However, in recent years the outlook has changed. More laboratories have reported reduced abnormality rates, as low as 1% (Soh et al. 2001; Maheran and Abu Zarin 1999; Ho 2000; Zamzuri et al. 2005), and this has given renewed confidence for clonal planting materials to go commercial on a full scale. The improvement observed is believed to be attributed to the introduction of a more suitable laboratory protocol coupled with stringent culling procedures at various stages of the tissue culture process (Maheran et al. 1995; Simon et al. 1998). However, the cause of abnormality still remains unknown. Based on the phenotype of the abnormal (mantled) flowers, where the stamen whorl is converted into a carpel whorl, it is not unlikely that changes in the expression of the MADS box genes specifying the affected flower whorls underlie the mantled phenotype. The MADS box directed differential display has proven to be an excellent method of isolating a broad set of members from the MADS box gene family expressed in a variety of tissues (Van der Linden et al. 2002, 2005). Through this technique 18 MADS box genes from the oil palm were isolated and characterised (Auyong 2006).

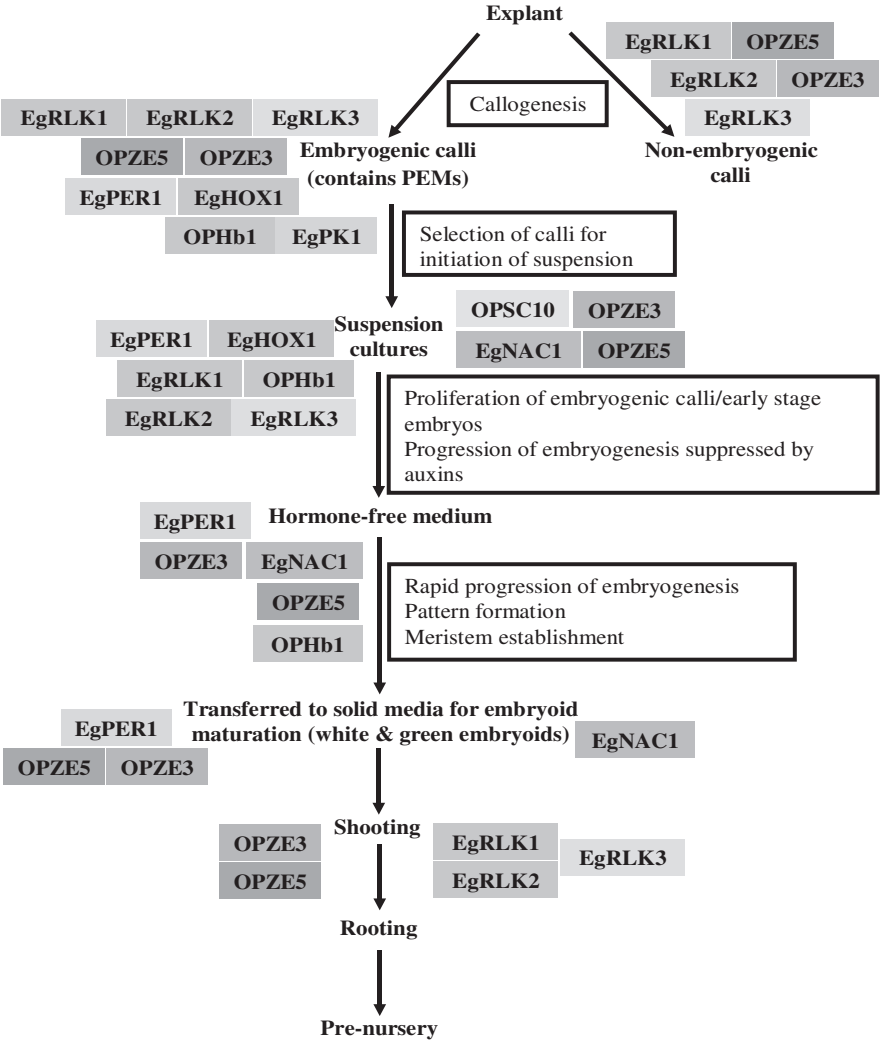


Fig. 11.9 Schematic representation of the expression profile of EgPER1, EgHOX1, EgPK1, EgNAC1, OPSC10, OPHb1, OPZE3, OPZE5, EgRLK1, EgRLK2 and EgRLK3 in somatic embryogenesis (PEMs, proembryogenic masses) (Source: Ong-Abdullah and Ooi 2006)

11.10 Genetic Engineering of the Oil Palm

As the oil palm planting materials are derived from a narrow gene pool, the introduction of new traits via conventional breeding techniques is restricted. The controlled pollinated behaviour in breeding of oil palm coupled with the long generation time (approximately 7–10 years) contribute to the slowness of conventional genetic improvement methods. Besides requiring large amounts of plant-

ing material, conventional methods are also imprecise and because most economically important traits are controlled by polygenic systems, the transfer of single traits is difficult. Almost 10 years of work is required to conclusively identify elite progenies.

Genetic engineering applied to the oil palm has the potential to

- reduce the time and cost of selecting desired mutants;
- improve the precision and restrict the amount of gene transfer;
- widen the genetic base of the oil palm.

MPOB embarked on research related to genetic engineering of oil palm as early as 1987. The focus of the project then was to engineer oil composition towards increased oleic acid at the expense of palmitic acid (Cheah et al. 1995). Palm oil with increased unsaturation will be useful for meeting consumer demands for mono-unsaturated and poly-unsaturated dietary oils. Higher unsaturation will also allow palm oil to penetrate the liquid or salad oil market. This programme served as a model for the genetic engineering of subsequent products.

Plants are the most efficient bioreactors and non-polluting factories. The oil palm, which is the most productive oil crop, can be considered as one of the most efficient green factories and its inherent high productivity can be channelled towards the development of high value products such as novel oils, nutraceuticals and pharmaceuticals. In addition to its high productivity, the oil palm has the advantage that it is a perennial crop. A palm will continue to manufacture the product of interest for 20–30 years thus making it an ideal candidate for genetic manipulation.

The key targets for modifying the oil palm are

- High oleic acid oil
- High stearic acid oil for confectionary end-use as cocoa butter substitute
- Ricinoleate rich oil
- Nutraceutical oil
- Palmitoleic acid rich oil
- High lycopene oil
- Biopolymers

11.11 High Oleate Transgenic Oil Palm

Palm oil with high oleic acid content was proposed for use as feedstock in the oleochemical industry. In order to modify oil palm for high oleic acid, the regulation of fatty acid biosynthesis in the oil palm needed to be understood. Palmitic acid is the predominant fatty acid in palm oil accounting for up to 44% of the total fatty acids. Oleic acid accounts for 39% of the total fatty acid composition. Biochemical studies were carried out to understand the cause for palmitic acid accumulation in the oil palm mesocarp. ß-ketoacyl-ACP synthase II (KAS II) and palmitoyl-ACP thioesterase were identified as the enzymes responsible for palmitic acid accumulation.

11.11.1 Biochemical Studies

KAS II enzyme was successfully isolated from oil palm mesocarp (Umi Salamah 1995). Increasing KAS II activity would increase the stearoyl-ACP pool that would subsequently be desaturated to oleoyl-ACP and finally released as in oleic acid by oleoyl-ACP thioesterase. The relationship between KAS II activity and the level of C18 unsaturation (C18:1 and C18:2) in oil palm was studied and a strong positive correlation demonstrated (Sambanthamurthi et al. 1996). Biochemical studies also showed that the level of C16:0 was negatively correlated to the level of C18:1. This negative correlation also demonstrated that palmitic acid accumulation was controlled by the activity of KAS II. Therefore, it was postulated that the higher the KAS II activity in the mesocarp the more palmitic acid could be directed through the pathway to produce oleic acid.

Thioesterase activity in oil palm was assayed against different acyl-ACP substrates using crude protein extracts of mesocarp tissues. The extracts showed maximum activity towards palmitoyl-ACP and to a lesser extent towards oleoyl-ACP. The thioesterases were partially purified and chromatography revealed that palmitoyl-ACP thioesterase and oleoyl-ACP thioesterase are two separate proteins (Sambanthamurthi and Oo 1990; Abrizah 1995). This is fortuitous as the activity of palmitoyl-ACP thioesterase could be safely down-regulated via antisense technology to reduce palmitic acid content without reducing the oleoyl-ACP thioesterase activity, which is required for accumulation of oleic acid. However, whether the excess oleoyl-ACP pool produced could be fully converted into oleic acid will be dependent on the activity of oleoyl-ACP thioesterase.

Desaturation of oleic acid is catalysed by oleoyl-CoA desaturase and requires CoA as a co-factor and takes place in the cytosol. Biochemical studies revealed that there is a strong correlation between KAS II activity and oleic acid and even higher correlation with linoleic acid (C18:2) (Sambanthamurthi et al. 1996). This implied that increasing the activity of KAS II would not only increase oleic acid but also linoleic acid. Therefore for production of high oleic acid oil palm, down-regulation of oleoyl-CoA desaturase was proposed as it could stop the spillover of oleic acid into linoleic acid (Sambanthamurthi et al. 1999, 2002).

11.11.2 Gene and Promoter Isolation

Following the confirmation of the regulatory role of the various enzymes in the synthesis of oleic acid, isolation of genes was carried out. Full-length KAS II clones were successfully isolated from oil palm mesocarp (Mohamad Arif et al. 2005). A full-length palmitoyl-ACP thioesterase gene was isolated and over-expressed in *E. coli* (Abrizah et al. 2000). The expressed enzyme was able to hydrolyze medium-chain and long-chain acyl-CoAs. Isolation of the oleoyl-CoA desaturase gene from oil palm has also been accomplished (Siti Nor Akmar et al. 2001).

Fatty acid manipulation is targeted to take place in the mesocarp, the fleshy part of the fruit where the edible oil is synthesised and accumulates. Therefore, the genes to be engineered either by up-regulation or down-regulation will be driven by a mesocarp specific promoter. Isolation of mesocarp-specific gene was therefore carried out followed by the isolation of its promoter (Siti Nor Akmar et al. 2001). The tissue-specificity of the promoter was verified by transient expression using the β-glucuronidase (GUS) reporter gene. Transient expression revealed expression only in the mesocarp slices and not in leaf discs.

11.11.3 Construction of Transformation Vectors for Producing High Oleic Acid Oil Palm

Based on biochemical studies, the strategy identified for increasing oleic acid was up-regulating KAS II activity and down-regulating palmitoyl-ACP thioesterase (Sambanthamurthi et al. 2000). Down-regulation of the oleoyl-CoA desaturase was also considered to avoid spillover of oleic acid to linoleic acid. By down-regulating oleoyl-CoA desaturase activity in soybean, researchers at DuPont increased oleic acid content in the seed (Broglie et al. 1997). The oleic acid content increased from 21.5 to 78.9% resulting in the reduction in linoleic acid content from 55 to 3%. Using the same strategy, oleic acid content in cottonseed oil was increased from 15 to 78% (Liu et al. 2000).

The use of more than one mesocarp-specific promoter to drive the different targeted genes may also be essential. Inserting many genes driven by the same promoter could lead to homology dependent gene silencing (Matzke et al. 1989) as there will be repeated copies of the same promoter sequences at the insertion loci.

Based on the proposed strategy, two sets of transformation vectors were constructed: (i) antisense palmitoyl-ACP thioesterase gene driven by CaMV35S promoter (Abrizah et al. 2000) and (ii) antisense palmitoyl-ACP thioesterase resistance and sense KAS II genes driven by mesocarp-specific promoter (Masani and Parveez 2004). Both constructs had the Basta gene for selection and both constructs were bombarded into oil palm embryogenic cultures. A few hundred plantlets have been obtained and transferred onto soil in a fully-contained biosafety greenhouse (Fig. 11.10).

As the numbers of transgenic cultures increase, it is important to screen the cultures and plantlets to avoid keeping the escapes and the transgenic lines without the gene of interest. Initial molecular analysis using PCR revealed that more than 90% of the transgenic lines were positive for the Basta resistant gene and also for the palmitoyl-ACP thioesterase gene (Parveez et al. 2003a). The PCR-positive cultures and plantlets were subjected to fatty acid analysis via gas chromatography. Fatty acid analysis on 100 transgenic oil palm embryoids that were transformed with antisense palmitoyl-ACP thioesterase gene revealed that some of the samples showed a reduction in palmitic acid and an increment in oleic acid composition as expected (Parveez et al. 2005). Further analysis is ongoing. A better understanding of the results is expected when the plants are mature and start fruiting.

Fig. 11.10 Transgenic plantlets in the fully-contained biosafety green house

11.11.4 High Stearate Transgenic Oil Palm

High stearic acid transgenic oil palm is expected to give rise to new applications such as the cocoa butter substitute and personal care products such as lotions, shaving cream and rubbing oils (Parveez et al. 1999). Biochemical studies revealed that oil palm contains an active stearoyl-ACP desaturase. Therefore, down-regulating the stearoyl-ACP desaturase activity could reduce the conversion of stearoyl-ACP into oleoyl-ACP. Using this approach, Knutzon and colleagues (Knutzon et al.1992) transformed rapeseed with an antisense copy of a stearoyl-ACP desaturase. The stearic acid content of the transgenic plants increased from 1.8 to 39.8% by weight in the seed as a result of concomitant reduction of oleic acid. The same was reported in cottonseed oil where stearic acid content was increased from 2 to 40% by down-regulating the same gene (Liu et al. 2000).

11.11.5 Construction of Transformation Vector for Producing Stearic Acid Oil Palm

The strategy identified for creating high stearate transgenic oil palm was down-regulating stearoyl-ACP desaturase in the mesocarp. A full-length clone of stearoyl-ACP desaturase was isolated from oil palm (Siti Nor Akmar et al. 1999). In addition,

down-regulating palmitoyl-ACP thioesterase and up-regulating KAS II in order to direct palmitoyl-ACP towards stearoyl ACP was also considered.

Initially, three transformation vectors were constructed carrying an antisense stearoyl-ACP desaturase gene driven by maize ubiquitin, CaMV35S and oil palm mesocarp-specific promoters. All constructs carried the Basta resistance gene for selection and were bombarded into oil palm embryogenic calli. Currently a few lines of Basta resistant polyembryogenic cultures have been obtained and are undergoing regeneration (Parveez et al. 2003b). It would be interesting to analyse the fatty acid composition of the cultures especially those that were transformed with genes driven by constitutive promoters. Furthermore, the morphology and growth of the transgenic plants would also be analysed later to evaluate the effect of constitutively increasing stearic acid content in the cells.

11.12 Production of Biodegradable Thermoplastics

Polyhydroxybutyrate (PHB) is the most common polyhydroxyalkanoate (PHA) produced as a storage material by bacteria under restricted growth conditions (Senior and Dawes 1973). PHB is synthesised in bacteria from acetyl-CoA by a sequence of three reactions catalysed by the following three enzymes: ß-ketothiolase, acetoacetyl-CoA reductase and PHB synthase (Anderson and Dawes 1990). PHA is an important polymer to be exploited for producing a wide range of environmentally friendly industrial polymers. Commercial production of PHA was initially carried out using bacterial fermentation. However, the production cost was 5–10 times higher than the cost of using petroleum based polymers (about $0.25–0.5/kg) (Poirier et al. 1995). Plant systems were proposed as they are capable of producing millions of tonnes of oils and starch at the lower price of $0.25–1.0/kg (Poirier et al. 1995) and it was envisaged that the existing pathways could be channelled towards the production of bioplastics.

The first successful synthesis of PHB in plant cells was demonstrated by Poirier and colleagues (Poirier et al. 1992) in Arabidopsis cytoplasm. In this study, reductase and synthase genes were used as the ketothiolase is available endogenously in plants. The highest amount of PHB synthesised was only 0.1% of dry weight. In another experiment, when PHB genes were transformed into cotton using a fibre-specific promoter up to 0.3% dry weight of PHB was synthesised in the cytoplasm (John and Keller 1996). Due to the low PHB synthesis, it was later proposed that higher PHB could be obtained by targeting the genes into plastid (Nawrath et al. 1994a). As fatty acid synthesis in plants occur in the plastid, highest flux of acetyl-CoA is expected to be in the plastid; therefore the highest amount of PHB may be synthesised in the plastid. As high levels of starch present in plastid do not affect its function, accumulation of PHB in plastid is not expected to interfere with plastid function (Nawrath et al. 1994a). When PHB genes were transformed into *Arabidopsis* plastid, PHB synthesis increased up to 14% of the dry weight (Nawrath et al. 1994b). The PHB granules were all contained in the plastids. When all the

genes were transformed on a single plasmid and targeted into plastid, the resulting transgenic Arabidopsis plants successfully synthesised PHB up to 4% of their fresh weight (Bohmert et al. 2000).

Besides producing PHB in cytoplasm, leukoplasts and peroxisomes were also targeted for synthesising PHB. Targeting the PHB genes into peroxisomes has resulted in accumulation of 3–7% dry weight of PHB in rapeseed (Houmiel et al. 1999). Hahn and colleagues (Hahn et al. 1999) later demonstrated the synthesis of 2% PHB per dry weight in the leukoplasts of Black Mexican Sweet maize.

Besides PHB, synthesis of a copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) or PHBV was also demonstrated in rapeseed and Arabidopsis (Slater et al. 1999). However, PHBV requires another substrate, propionyl-CoA. As shown in Fig. 11.10, threonine deaminase (ilv) or threonine deydratase (tdcB) genes are required to produce propionyl-CoA and another type of ß-ketothiolase (bktB) is needed to convert propionyl-CoA to ß-ketovaleryl-CoA for producing hydroxyvalerate (PHV). Using the bktB, phbB, phbC and ilv genes targeted to plastid under the control of a constitutive promoter, they were able to synthesise PHBV up to 1.6% of dry weight in Arabidopsis. When a seed-specific promoter was used, up to 2.3% of PHBV (dry weight) was synthesised in rapeseed.

11.12.1 Strategy for Synthesising PHB or PHBV in Transgenic Oil Palm

MPOB is collaborating with the Massachusetts Institute of Technology (MIT) for the metabolic engineering of oil palm for the production of biodegradable plastics (PHB and PHBV). The oil palm is the most productive oil crop known. The starting substrate for oil synthesis is the same as that for PHB synthesis, i.e. acetyl CoA. This implies that the oil palm is the best crop for metabolic engineering to produce PHB since the oil palm has a very high flux for acetyl CoA. This intervention strategy is to channel lipid biosynthesis towards bioplastic synthesis instead. Since acetyl CoA carboxylase catalyses the first committed step in fatty acid biosynthesis, this gene will be silenced in an effort to stop or reduce lipid biosynthesis. We have isolated β-ketoacyl ACP synthase and acetyl CoA carboxylase genes from oil palm mesocarp. The other genes (PHB and tdcB) have to be transferred from bacterial sources and we received these from MIT.

Besides the relevant genes for bioplastic production, an oil palm ACP gene transit peptide (Rasid et al. 1999) and tobacco RB7 matrix attachment region (MAR) (Matzke and Matzke 1991) were also included in the constructs for plastid targeting and stabilising transgene expression, respectively. The MAR is essential as multiple copies of mesocarp-specific promoters will be used to drive three or five genes for PHB or PHBV in order to minimise homology dependent gene silencing (Matzke et al. 1989). For the production of PHBV, threonine deydratase (tdcB) (Guillouet et al. 1999) was also used for producing propionyl-CoA substrate. Mesocarp was targeted because it has the highest acetyl-CoA pool.

Four transformation vectors carrying the relevant PHB and PHBV genes driven by constitutive or mesocarp-specific promoters have been successfully constructed (Masani et al. 2001; Greg et al. 2001; Masani and Parveez 2003) and transformed in the oil palm. To date, regeneration of Basta-resistant calli has resulted in a few hundred plantlets for the ubiquitin constructs and have been transferred onto soil in the biosafety nursery. Initial PCR analysis revealed that more than 90% of the transgenic lines were positive for the Basta resistant gene and also for the PHBV or PHBV genes (Parveez et al. 2003c). The PCR positive cultures and plantlets were later subjected to PHB or PHBV analysis via HPLC to examine whether there are any PHB or PHBV synthesised in the cultures or plantlets. The results showed that there is a peak corresponding to PHB (Fig. 11.11). Further analysis and quantification of PHB content is ongoing.

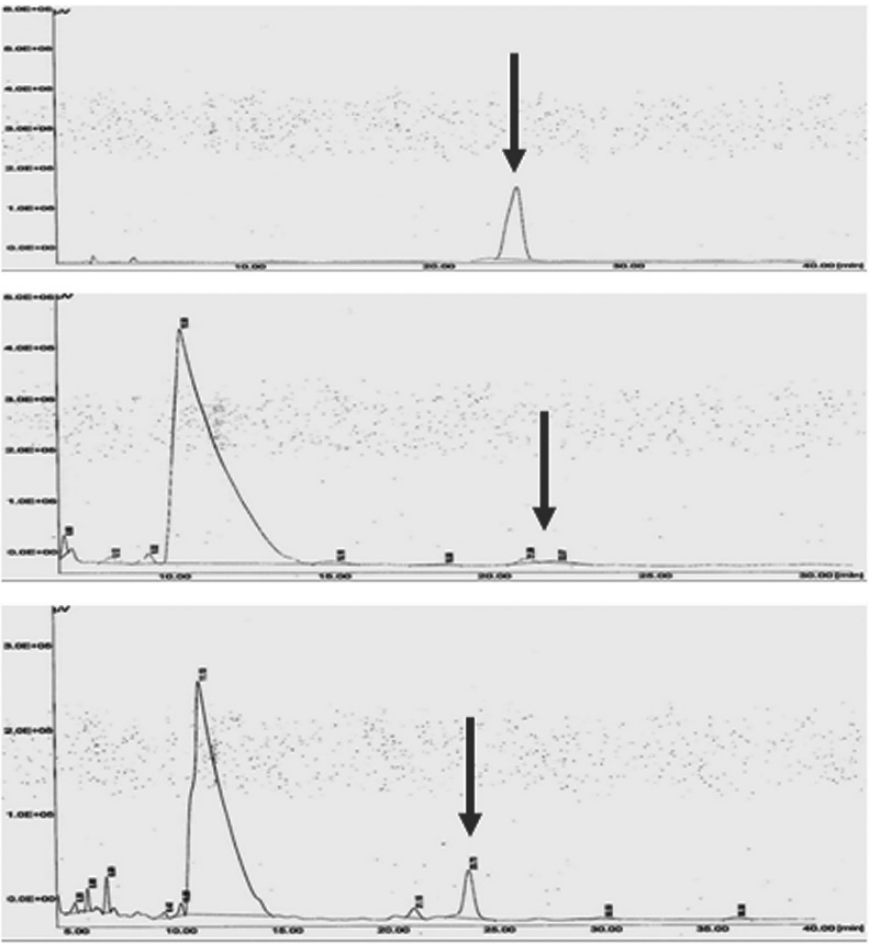


Fig. 11.11 HPLC profile for standard PHB (*top*), non-transgenic oil palm (*middle*) and transgenic oil palm synthesising PHB (*bottom*). Arrow indicates the crotonoic acid derived from PHB (*See* Color Insert)

11.13 Containment

The oldest transgenic palms, which are eight years old, are still expressing the Basta-resistant gene and have started fruiting in a fully-contained biosafety greenhouse. The biosafety greenhouse is covered with a size 50 mesh (50 holes per square inch) and fixed with a double layer door to trap and prevent insects and small animals from entering the greenhouse during opening and closing. Entrance to the greenhouse is limited to trained personnel only. Leaves, roots and any parts of the transgenic palms removed are safely collected and autoclaved in autoclavable bags prior to appropriate disposal.

11.14 Future Directions in Genetic Engineering

Besides all the interesting research activities carried out and precautions and risk assessments taken to ensure safety of the transgenic oil palm, alternative approaches have been initiated to further ensure the biosafety of transgenic oil palm. Concern over the potential hazard of transgenic plants to the environment and human health has called for the creation of antibiotic marker-free transgenic crops (Daniell 1999). There are three potential approaches for developing marker-free transgenic plants: elimination of marker genes, replacement of the marker genes, self-containment of the transgenes. Elimination of marker genes could be carried out by either using two T-DNA plasmids (Komari et al. 1996) or using systems such as Multi Auto Transformation Vector System (Ebinuma et al. 1997). Replacement of antibiotic marker genes with more friendly genes such as phosphomannose isomerase gene (Joersbo et al. 1998) may be investigated. Finally, self-containment of the transgenes could also be achieved via systems such as chloroplast transformation (Daniell et al. 1998). Another concern about transgenic plants is the integration of plasmid or vector DNA into the plant genome besides the gene of interest. This has been demonstrated in direct gene transfer (Kohli et al. 1999) and Agrobacterium-mediated transformation (Cluster et al. 1994). A group at John Innes Centre (UK) has transformed rice with three minimal transgene cassettes (promoter, open reading frame and terminator) and demonstrated simple and low-copy-number integration events as compared to the use of supercoiled or linearized plasmid (Fu et al. 2000). This is a technically feasible approach to be considered for oil palm.

11.15 Conclusions

Conventional breeding and selection has resulted in tremendous improvement in yield and quality of oil produced by the oil palm and will continue to play a pivotal role in crop improvement. The oil palm, which has a long breeding cycle and requires large tracts of land for breeding trials, is ideally suited to the genomics approach for enhancing its value. Whole genome analytical techniques such as genetic mapping and ESTs have the potential to improve selection methods and

(*See* Color Insert)

reduce the time required to bring new varieties into the market. The advances in genomics, such as DNA Microarray are also expected to revolutionise research in oil palm genetics. Agronomic traits such as efficiency in nutrient utilisation, stress tolerance and disease resistance are under the control of many genes. In breeding, it is recognised that interaction between genotype and environment plays an important part in determining the yield potential of selected varieties. Yield itself is controlled by many genes. Under such circumstances the ability to view the performance of many genes all at the same time is thus a dream come true for agricultural plant improvement. It is conceivable that in the future, diagnostic chips will become common tools to predict for agricultural productivity in oil palm. Biotechnology's greatest challenge to acceptance by society is the public perception of the benefits and detriments of GM products. Owing to the long time to bearing of the oil palm and the need for stringent field evaluation over several years, commercialisation of GM oil palm is not anticipated until at least 2020. While MPOB has the technology and a strong scientific base for producing a whole spectrum of high value products via genetic engineering, the eventual commercialisation of this technology will depend on market acceptance and demand. GM products are no different from any other product that is marketed. The concept of satisfying the wants, needs and demands of the customer applies. Currently, palm oil is GM-free.

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Chapter 12
Olive Breeding

Andrea Fabbri, Maurizio Lambardi, and Yelda Ozden-Tokatli

Abstract The olive (*Olea europaea* L.) is, at the same time, one of the most ancient domesticated fruit trees and the most extensively cultivated fruit crop in the world, covering an area of about 7.5 million hectares. The recent diffusion of olive outside its traditional area of cultivation, the Mediterranean basin, together with a continuous trend in the modernisation of its industry, has greatly increased in recent years the demand for improved cultivars by olive growers. Hence, programmes of clonal selection and cross-breeding have been started in the main olive growing countries, aiming at selecting genotypes characterised by early bearing, resistance to pests and to abiotic stresses (such as frost and drought), limited alternate bearing, suitability to intensive culture and to mechanical harvesting, as well as high-quality productions, in terms of both organoleptic characteristics of fruits and oils, and high contents in substances useful for human health. This chapter reviews the recent advances in olive breeding, providing extended information on flower biology, main world cultivars, germplasm collection and preservation, propagation techniques, main characters for olive improvement and traditional breeding techniques (clonal selection, cross breeding and mutagenesis). In addition, information on the recent developments of olive biotechnology for the improvement and the safeguard of genetic resources (tissue culture, synthetic seed technology, genetic transformation and cryopreservation) is also reported.

12.1 Introduction

The olive (*O. europaea* L.) is one of the most ancient domesticated fruit trees and its products have been valued since ancient times. The oil extracted from the fruit mesocarp is a valuable and healthy food, but in ancient times its importance was also due to other uses, such as lamp fuel, wool treatment, medicine and cosmetic, soap production and the like. As a food, it is used for salads, for cooking and to preserve

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Table 12.1 Average world production of olive (2000–2005) according to the International Olive Oil Council (http://www.internationaloliveoil.org/)

	Olive oil			Table olives	
	($\times 10^3$ t)	%		($\times 10^3$ t)	%
World	2739.3	100.0		1541.9	100.0
EU countries	2171.1	79.3		681.8	44.2
Other countries	568.2	20.7		860.1	55.8
Leading countries					
Spain	1051.3	38.3	Spain	497.9	32.2
Italy	683.1	24.9	Turkey	161.5	10.5
Greece	394.2	14.3	Egypt	136.7	8.9
Tunisia	142.8	5.2	Syria	134.2	8.7
Turkey	112.3	4.1	Greece	101.7	6.5

other foods. Table olives are also a typical component of the Mediterranean diet and are consumed after processing and pickling in different ways.

The olive originated in the Mediterranean area and the countries bordering the sea still produce 97% of the world oil production. The main producers are Spain, Italy and Greece for olive oils and Spain and Turkey for table olives (Table 12.1). In addition, olive culture is expanding to many other countries outside the Mediterranean basin, such as the United States (California), Australia, China, South Africa, as well as in other sub-tropical and warm temperate areas, usually in fairly arid regions and on well-drained soil. Today, olive is the most extensively cultivated fruit crop in the world, covering an area of 7.5 million of hectares (http://faostat.fao.org/).

The earliest signs of olive cultivation can be traced back to 4 B.C. and before (Zohary and Spiegel-Roy 1975; Loukas and Krimbas 1983; Zohary and Hopf 1994) to areas of the Eastern Mediterranean coasts and islands, although the ancestors of currently grown olive cultivars are still believed to have been domesticated in the mountainous territory, south of the Caucasus, covering today's western Iran, Eastern Turkey, Lebanon, northern Israel, Syria and northern Iraq. From the Eastern Mediterranean the olive moved westwards to Greece and the Aegean archipelago, although Crete and Cyprus probably belong to the oldest olive centre of diversity. In these areas, collectively considered a secondary centre of diversity, the olive grew in importance and underwent further selection by humans, in the period between 3 and 2 B.C. In Crete, in 16 B.C., there existed in Knossos a huge deposit of clay jars (still visible today in the excavation site) able to store five times the amount of oil the local population could consume in 1 year, thus indicating a well-developed trade in olive oil.

Around the beginning of 1 B.C., a second migration appears to have taken place, again westwards, to Sicily and Tunisia, an area regarded as olive's tertiary centre of diversity. From there, around 600 B.C., probably through Etruria (today's Tuscany), the crop is reported by the classical historians to have reached the Romans. Up to this point the olive had moved slowly westwards, first on the ships of Phoenician merchants and later on those of Greek colonists. These peoples had spread the crop in many other places of the Mediterranean Sea including Spain, France

and North Africa with varied results. But the conquest of the whole area by the Roman legions and its transformation into a vast, united empire, made trade and communications far more intense. The olive benefited from this situation, and the Romans spread its cultivation in new areas or favoured it in places where it stagnated, especially when Italy appeared unable to provide the required supply of olive oil. The crop achieved its maximum economic importance in the II–III centuries A.D., particularly in Northern Africa, but also in Spain, Dalmatia and the French Provence. With the fall of the Roman Empire information about the olive becomes scarce. Its cultivation dropped dramatically with the reduction in population and the abandonment of large areas that took place in the course of the early Middle Ages. This was not the case in the territories under Arab rule, where the crop remained important to the point that its cultivation was forbidden in Sicily, in order to protect production in North Africa, probably the main producer at the time (Acerbo 1937).

In Europe, olive oil acquired new importance only in XVI–XVII° centuries, when it became a significant trading commodity for Venetians who imported it from their Aegean possessions, such as Crete and Cyprus. Thus, slowly olive plantations began to spread in the Mediterranean areas where they can still be found today, with the exception of most of North Africa, where it was reintroduced on a large scale much more recently.

The arrival of the olive in the Western and Southern hemispheres is recent. Argentina, California, Australia and South Africa, where the enthusiasm for the crop of Mediterranean migrants had ensured its introduction across the last century, all proved to have suitable environments for olive commercial cultivation.

12.2 Botany of *Olea europaea* L.

12.2.1 Vegetative Structures

Olive is a long-living evergreen tree. In suitable environments millenary trees are not uncommon. The wood resists decay and the tree, if destroyed by adverse events, can easily regenerate from suckers (‘pollards’), which are abundantly produced by roots and by particular structures located around the collar, the ovules.

The cultivated olive is a medium-size tree, 4 to 8 metres high, according to cultivar and management. The trunk has a smooth surface in the young tree that soon becomes rough and twisted. The canopy, if unpruned, tends to form a dense globe. The shoots are slender and may carry either leaf buds or flower buds at leaf axils; mixed buds are rare but can occur. Seedlings have a long juvenile stage and the tree is traditionally propagated by cutting or grafting (see Section 12.4.1.). Juvenile structures, which at times are seen as suckers produced by the rootstock, have shorter internodes, smaller and darker leaves and can be thorny. The leaves are thick, persistent (may last 2–3 years), oppositely arranged. The upper surface is strongly cutinized and the lower surface, where stomata are, is covered by a thick felt of peltate hairs that gives it a silvery appearance. The root system, in

spite of the marked resistance of the tree to drought, is relatively shallow, although soil characteristics and management have a major influence on its distribution (Rapoport 1999).

12.2.2 Flower Biology

12.2.2.1 Inflorescence Formation and Flowering

Flower bud inflorescences are borne at leaf axils (hence a maximum of two per node). Usually flower buds are formed on the shoots developing the year before anthesis. The formation of flower bearing buds is a process requiring the passage of the meristematic apex of the bud, undifferentiated in its early stages of growth, to a structure carrying flowers. Flower differentiation takes place in winter (that is, in the Northern Hemisphere, in the period from late February to mid-March) although in some areas it may last longer. The timing and extent of flower differentiation seem to depend on the achievement of specific chilling requirements, as low winter temperatures influence not so much floral evocation as rather the expression of a flowering potential already determined in warmer periods. As a rule, floral differentiation occurs during the 40–60 days before anthesis and the process is completed as the inflorescence emerges and develops (Fabbri and Benelli 2000).

Inflorescence development begins in early spring, roughly one month after the onset of flower differentiation, usually starting on the south side of the tree (in the Northern Hemisphere). It is gradual, and the time between inflorescence emission and anthesis is usually around 4 weeks, up to 6 or more in warmer climates, in separate flushes. As a rule, the earlier the emission of inflorescences, the higher the expected production, as fruit set may take place in less dry conditions. However, environmental events may markedly alter the forecast. Flowers are usually borne on 1-year-old shoots. Only occasionally inflorescences develop on 2- or 3-year-old branches.

Two types of flowers are present each season: ‘perfect flowers’, containing stamens and pistils, and ‘staminate flowers’, containing aborted pistils and functional stamens. ‘Ovary abortion’ refers to absence of ovary or to small, imperfect, non-persistent ovary. All olive trees display ovary abortion, although at different extents, depending on cultivar, environment, year, inflorescence and type of shoot. Its incidence is variable, from less than 10–70% to more. In spite of that, production is usually not depressed as normal harvests require no more than 4% of fruit set. One-hundred percent abortion cannot exist in a commercial cultivar, with the exception of ‘Swan Hill’, an ornamental cultivar, selected by Hartmann in Australia (Hartmann 1967), which displays this character advantageous for olive trees utilised in urban forestry or as ornamentals.

12.2.2.2 Anthesis and Pollination

Full bloom occurs in full spring (e.g., May in warm areas such as California, Southern Italy, Greece and Spain; at higher altitudes and elevations full bloom is

delayed up to mid-June). Differences can be observed among cultivars, which are to be kept into account when selecting pollinators. Anthesis normally lasts 2–3 days on individual inflorescences and 5–6 days on the individual tree (up to 10–15 days if temperatures are relatively low). A flower is fully opened when both anthers and petals are separated. During the hottest part of the day anther dehiscence takes place and an abundant amount of pollen is shed.

The amount of pollen produced appears to be a varietal characteristic: for example, 'Leccino' and 'Frantoio' (two oil cultivars very common in Central Italy) produce small amounts of pollen, and larger quantities are produced by 'Ascolana', 'Manzanilla' and 'Pendolino'. More important is the pollen's ability to germinate: this characteristic appears to fluctuate (in vitro) between 12 and 60% (Zito and Spina 1956; Fernandez-Escobar et al. 1983).

Pollination is influenced by several factors, the most important being (Fabbri et al. 2004):

- temperature, which has the effect of enhancing tube growth, although, when too high, the stigma may get dry. For anther dehiscence the optimum is 30°C with 50% of Relative Humidity (RH). A good value of RH also enhances pollen germination;
- rain, which is always negative; indeed, it may determine pollen grain plasmolysis, dilute stigma secretions and hinder pollen transport;
- wind, which is fundamental for this anemophilous species. When too strong, the wind may transport masses of pollen away from the grove. Although olive pollen can be found as far as 12 km from the tree, the effective range is considered to be not larger than 30 m.

12.2.2.3 Sterility

Sterility may be due to factors different from those affecting ovary abortions, such as anomalies during meiosis producing imperfect gametophytes ('cytological sterility'), quite rare in olive, and incompatibility ('factorial sterility'). Incompatibility occurs when a perfect pollen grain fails to germinate on the stigma or germinates, but its tube growth is somehow impeded. Incompatibility may be between two cultivars ('inter-' or 'cross-incompatibility') or when a cultivar is genetically programmed not to be fertilised by its own pollen ('self-incompatibility').

'Self-' and 'cross-incompatibility' mechanisms are both common in olive and have been the main reason for the large genetic variability typical of the species. This aspect has been extensively studied due to its recognised importance in the establishment of olive orchards (to assure inter-compatibility among cultivars and, hence, productivity) as well as when breeding programmes are designed. Notwithstanding, contradictory results have been often reported as the behaviour of some of the main important cultivars (e.g., 'Arbequina', 'Frantoio', 'Manzanilla de Sevilla' and 'Picual') resulted, according to different studies, either 'self-incompatible' or 'self-compatible' (Diaz et al. 2006a). More investigation seems necessary on this aspect particularly to ascertain the genetic control of the 'self-' and 'cross-compatibility' mechanism in olive.

12.2.2.4 Fruit and Seed Development

In olive, the average final fruit set (i.e., the ratio between number of fruits persisting until maturity and the initial number of flowers) is around 2%, although yearly fluctuations can be wide. Fruit drop is utilised by the plant to adapt production to its elaborating surface. Other factors may influence fruit drop, such as nutritional and water deficiencies, weather conditions during bloom, sterility, lack of pollinators and pests.

The olive fruit is a drupe, which means it is made of two main parts, that is, pericarp and seed. The pericarp is made of (i) the skin (exocarp), free of hairs and with stomata, (ii) the flesh (mesocarp), the tissue containing oil, and (iii) the pit (endocarp), a lignified shell enclosing the 'true seed'. The pit and the contained seed are the olive 'stone'. The seed consists in a seed coat and a thick endosperm that ensheath a large embryo, made of flat cotyledons and of short radicle and plumule. As a rule there is only one seed per fruit, rarely two. In some Spanish cultivars the occurrence of nucellar polyembryony has been reported. Fruit shape and size, pit size and surface morphology vary greatly among cultivars, and are the most reliable morphological features to distinguish between cultivars.

The embryo makes up for most of the seed volume. The seed coat, derived from the integuments that represented the main ovular tissues, is thin, leathery and rich in vascular ridges. Between seed coat and embryo is a layer of endosperm, rich in starch (King 1938). The embryo has two quite evident large cotyledons, the embryonic leaves. A short radicle, located at the lowest end of the embryonic axis, will give rise to the root system. Between the cotyledons is a small plumule from which the future epigeic system (i.e., the plant parts that will be exposed to the open atmosphere) will develop. The embryo is usually completely formed after five months from full bloom. No further morphological or anatomical changes appear to occur in the embryo, although dormancy is imposed on the seed later in the season. Seed growth means a gradual embryo enlargement, which at the end occupies most of the space inside the endocarp at the expense of the endosperm.

Usually pollination and fecundation are essential for fruit set and early seed development. The presence of a vital seed in a growing drupe is not essential for fruit development. Indeed, many apparently normal fruits have no seeds. The fruit can also develop without the presence of a fertilised ovule (parthenocarpy), but in this case the fruit remains distinctly smaller.

12.3 Genetic Resources

The latin scientific name of the cultivated olive is that given by Linnaeus in 1764, *O. europaea* L. The latin name of the genus is believed to derive from the Greek word *elaion* (oil), while the name of the species underlines its European (or, better, Mediterranean) distribution. The Mediterranean climate is characterised by mid-season rains, dry summers and winters, a short-lived cold season with occasional frosts. So typical is the olive of such climate that it is the species better suited to

define a climate as Mediterranean. Few other areas in the world possess similar climatic features and olive culture has been introduced recently in many of them.

The cultivated olive belongs to the family Oleaceae. The family, made up of more than 30 genera and over 500 species, is distributed in tropical and temperate regions of the world. The genera include some ornamental and agricultural plants, such as *Forsythia, Fraxinus, Osmanthus, Jasminum, Ligustrum* and *Syringa*. The fruits of the Family are drupes (*Olea* spp.), berries (*Jasminum* spp.), capsules (*Syringa* spp.) or samaras (*Fraxinus* spp.). *O. europaea* is the only species of the genus *Olea* that can be found in the Mediterranean basin and the cultivated olive originated from the wild form 'oleaster' that is still present in most coastal areas (Zohary and Hopf 1994; Fig. 12.1). All the other species (over 30) are distributed in subtropical to warm temperate areas of both the Northern and Southern Hemispheres (Bartolini and Petruccelli 2002).

12.3.1 Wild and Cultivated Species

Within the cultivated *Olea* two subspecies are distinguished: *O. europaea* L. subsp. *sativa* (Loudon) Arcangeli (= subsp. *europaea*), to which belong the numerous cultivated varieties, and *O. europaea* L. subsp. *oleaster* (Hoffm. & Link) Negodi (= subsp. *sylvestris*) (Miller) Hegi, to which belong the spontaneous forms commonly called 'oleasters' (Ciferri 1950; Zohary 1994). The relationship between the cultivated varieties and the wild forms has been the object of many hypotheses. The ancestor of cultivated olive is supposed to be the wild form, easily distinguished by thorny shoots, small and roundish leaves, small and elliptical fruits with a thin oily mesocarp (Rugini and Lavee 1992; Zohary 1994; Amane et al. 1999). The domestication of the oleaster goes back to 4,000–3,000 B.C. when mass selection of trees started, presumably choosing the trees whose fruits were larger and richer in oil.

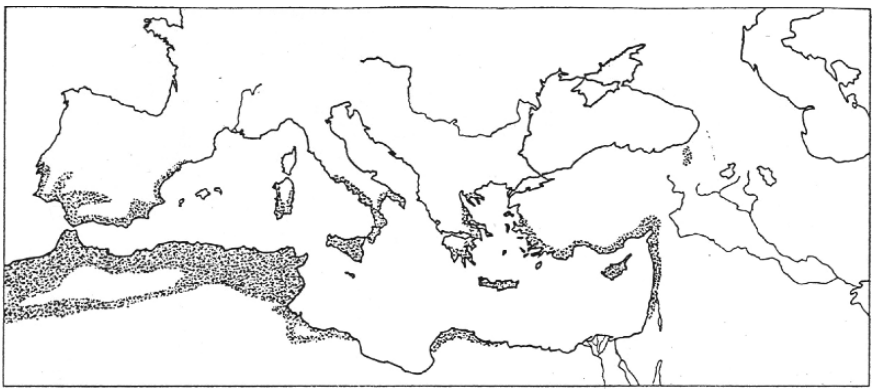

Fig. 12.1 Distribution of the wild olive (*O. europaea* subsp. *oleaster*) is the progenitor of the cultivated olive. This ancient form of the species is still present in several coastal areas of the Mediterranean basin (from Zohary and Spiegel-Roy 1975)

The selection was made possible by the easy vegetative propagation of the species with traditional techniques.

The botanical origin of the olive is not completely clear, and several *Olea* species have been considered as possible ancestors. A marked morphological similarity exists between spontaneous and cultivated forms of *O. europaea*, and species growing outside the Mediterranean basin – such as *O. chrysophylla* Lam. (= *O. africana* Miller), diffused in Africa and Asia, *O. excelsa* Ait. of the Canary Islands and *O. ferruginea* Royle (= *O. cuspidata* Wall.), native of Central Asia – have been indicated to be the ancient parents of the olive (Chevalier 1948). For Ciferri and Breviglieri (1942) and for Vavilov (1951) the ancestors could have been *O. Laperrini, O. chrysophylla* and *O. cuspidata*. Abundant data is being produced by molecular analysis of *Olea* genomes and more light is expected to be shed on the origin of the species (Ganino et al. 2006a).

Cultivated olives are nearly all diploid with $2n = 2x = 46$; occasional triploids and tetraploids have been reported as well as one case of polysomaty ($2n = 55$). The chromosome number of the species was first established by Breviglieri and Battaglia (1954) whose observations on chromosome morphology led them to assume that the species had originated by allopolyploidy, probably by parents whose haploid chromosome numbers were $n = 11$ and $n = 12$. These chromosomal numbers are present in several species of the Oleaceae Family. The hybrid is sterile and unstable, and its survival must have been granted by duplication of the genome. With reference to the centromere position, the chromosomes are of the median and sub median type. Three pairs of satellite chromosomes have been identified, smaller than in other species (Falistocco and Tosti 1996).

12.3.1.1 Characterization of the Cultivars by Morphological, Biochemical and Molecular Markers

Due to its early domestication and its large spread in the Mediterranean basin, the olive is particularly rich with cultivars, with a large number of synonyms and homonyms that make their description and classification particularly difficult. Cultivars are better defined as 'cultivar-populations' as they generally comprise clones that are separated by a number of minor characters (Morettini 1954a; Scaramuzzi and Roselli 1986; Roselli 1990). A detailed study has listed over 1,200 cultivars originating from 34 countries and preserved worldwide in 79 collections, with over 3,200 synonyms (Bartolini, Prevost, Messeri and Carignani 1998). More than 800 cultivars are for oil production, over 100 are table olives and the remaining are used for dual purpose.

Many botanists tried to describe olive cultivars without resulting in an accurate classification (e.g., Prevost and Mostardini 1999; Ganino et al. 2006a). At the onset of the nineteenth century, Simòn de Rojas Clemente (in Barranco and Rallo 1984) and Tavanti (1819) classified the olive cultivars depending on leaf, fruit and endocarp characteristics. Based on these three morphological characteristics, Ruby (1917) evaluated the differences among French cultivars. However, with the necessity to evaluate biological and ecological characteristics, in addition to the

morphological ones, Ciferri et al. (1942) made a morpho-ecological classification of olive. Using these numerous morphological and biological characters, a very detailed descriptor list was prepared for 70 olive cultivars grown in central Italy. Although the system had some limitations, the list soon became a fundamental reference for scientists. Afterwards, Barranco and Rallo (1984) prepared a pomological list (including characters of fruiting branches, leaves, inflorences, fruits and endocarps) to identify Andalusian olive cultivars. With the aim of making uniform the methodology of germplasm data collection, another descriptor list was prepared in 1985 under the supervision of Union Internationale pour la Protection des Obtentions Végétales, Geneva (UPOV). Other descriptor lists worthy of mention have been, in the following years, those of Leitão et al. (1986), Cimato et al. (1997) and Rallo et al. (2005).

The complexity of classification and the drawbacks of the morphological markers, which can be affected by the environment and the plant developmental stage, was the reason for the development of biochemical (isozymes and allozymes) and molecular (e.g., RFLP, RAPD, AFLP, SSR) markers for cultivar characterisation. A first attempt for the discrimination among olive cultivars by biochemical markers (i.e., the isozyme analysis of the olive pollen) dates back to the early 1980s (Pontikis et al. 1980). Following investigations demonstrated that biochemical markers, based on the detection of polymorphisms in enzyme protein composition, were suitable to characterise olive germplasm (Trujillo et al. 1990; Potes et al. 1999). A drawback in the use of pollen for varietal identification lay in the fact that sample collections were possible only at specific periods of the year (Trujillo et al. 1999). Analyses of phenolic content (Heimler et al. 1994) and of seed storage proteins (Durante et al. 1992) have been other attempts made in olive for the biochemical identification of cultivars. However, in time it was evident that these approaches were insufficient for a clear discrimination of plants. The reason was due to the limitations of isoenzymatic analyses, such as (i) only a small part of the structural genes is represented, (ii) nucleotide alterations are often undetectable because not all nucleotide substitutions result in a variation of amino acids, and thus at the protein level, and (iii) isozymes produced as a result of transcription and translation are regulated by several factors, including origin and physiological stage of tissues and environmental conditions, and thus they present scarce reproducibility.

Development of molecular markers revolutionised genetic analysis of plant genomes as they are free from environmental influence and have potential to identify variations at the DNA level. Restriction Fragment Length Polymorphism (RFLP) markers have been used to distinguish wild olives from cultivated varieties, confirming the Mediterranean basin as the site of olive domestication (Besnard et al. 2001; Besnard et al. 2002). They also permitted the analysis at molecular level of 95 plants obtained by the crossing of ‘Leccino’ with ‘Dolce Agogia’ (De la Rosa et al. 2003).

The Random Amplified Polymorphic DNA (RAPD) analysis, based on the Polymerase Chain Reaction (PCR) technique, does not contain the technical inconveniences of RFLP markers as only a very small amount of genomic DNA (25–100 ng) is sufficient, and the process is relatively fast and simple. This technique

has been applied successfully to identify olive cultivars from several countries (Fabbri et al. 1995; Cresti et al. 1996; Durante et al. 1999; Mekuria et al. 1999; Belaj et al. 2001; Ergulen et al. 2002; Wu et al. 2004; Ganino and Fabbri 2005). In spite of their wide use for olive cultivar characterisation, RAPDs are dominant markers and thus cannot differentiate homozygote from heterozygote, hence limiting their potential to be used directly as a selection tool for desirable traits in breeding programmes.

The combination of enzymatic digestion of DNAs and selective amplification of fragments has favoured the use of Amplified Fragment Length Polymorphism (AFLP) markers, which have high reproducibility and wide applications in cultivar identification, germplasm analysis and genetic mapping. AFLP markers have been used to determine genetic similarities and/or polymorphisms among the different forms of *Olea* (Angiolillo et al. 1999; Sanz-Cortéz et al. 2003; Montemurro et al. 2005; Owen et al. 2005; Grati Kamoun et al. 2006). AFLP markers have also been used jointly with RAPDs to study the presence of intra-cultivar variability (Belaj et al. 2004) as well as with SSRs (Bracci et al. 2006a; Montemurro et al. 2006)

The Microsatellites or Simple Sequence Repeats (SSRs) are the most recent and promising molecular approach to olive cultivar identification. The SSRs are codominant, easily reproducible, randomly and widely distributed in the genome, characteristics that make them very useful in plant breeding programmes. In a specific study on genetic diversity and relationships among 32 Italian and Spanish cultivars, SSRs showed the highest level of polymorphism and provided more information than AFLPs and RAPDs, although AFLPs was the technique revealing the highest number of bands per reaction (Belaj et al. 2003). In a few years, the application of SSRs to olive has been impressive and several groups have already given important contributions to discriminate among olive cultivars (Rallo et al. 2000; Sefc et al. 2000; Bandelj et al. 2002; Lopes et al. 2004; La Mantia et al. 2005; Bracci et al. 2006b; Trujillo et al. 2006; Diaz et al. 2006b; Ganino et al. 2006b). In addition, important information is also expected from the applications of the SSR technique to the mapping and breeding of olive genome (Cipriani et al. 2002; De la Rosa et al. 2004; Wu et al. 2004) also to construct genomic linkage maps of olive and to allow for early selection of progenies according to their growth and fruiting characteristics (‘marker-assisted selection’). The technique has been used as well to determine the varietal composition of olive oils through the analysis of DNA extracted from the oil (Breton et al. 2004; Testolin and Lain 2005). The major drawback of the analysis lies in the time and cost required for SSR isolation.

Other molecular markers such as Sequenced Characterised Amplified Region (SCARs) and Inter-Simple Sequence Repeats (ISSR) have been used to a lesser extent in olive (Hess et al. 2000; Gemas et al. 2004; Busconi et al. 2006; Essadki et al. 2006). However, a promising research is in progress on single nucleotide polymorphisms (SNPs) in a fragment of phytochrome A gene of olive, using high-resolution DNA melting analysis to simultaneously scan mutations and genotypes with unlabeled probes (Muleo et al. 2006).

12.3.1.2 Main World Cultivars

Spain, the olive leading country with the highest table olive and oil production, has 183 olive cultivars (Bartolini et al. 1998), among which 'Sevillana' and 'Manzanilla' are known as the most important table cultivars. The former has large and golden-yellow coloured fruits with a flesh/stone ratio of 7.5:1. A clone of 'Sevillana', the 'Spanish Sevillana', is also grown in Algeria. The cv Manzanilla, grown worldwide, has medium size, symmetrical and apple-shaped fruits with green skin including tiny whitish spots. In addition to table olive cultivars, Spain has many important cultivars that are mainly used for oil production. Among them, 'Picual' is grown on almost 1/3 of the whole planted surface in Spain, and is therefore one of the most cultivated varieties in the world. It has an elongated and nearly symmetrical fruit with a medium/high oil content (22–23%). 'Arbequina' is another important oil cultivar of Spain, which is not only grown in Catalonia (North Spain), but also widely used for new plantations in Argentina and Chile; its suitability to mechanical harvesting is increasing its importance worldwide. The small, generally round shaped fruits of 'Arbequina' contain 20–21% of oil under non-irrigated conditions. Besides 'Picual' and 'Arbequina', 'Hojiblanca', which is grown mainly in the Cordoba district of Andalusia, has a medium content (17%) of good quality oil (Barranco 1999) and can be also used as a table olive. Other important oil cultivars are 'Cornicabra' and 'Morisca'.

Italian olive culture is characterised by an extremely high number of cultivars due to the earliness of introduction of the species, the variety of environments that olive finds in the country and the political fragmentation of its territory in the past centuries. As many as 538 cultivars, with over 1,300 synonyms, are reported (Bartolini et al. 1998). 'Frantoio' is one of the major oil cultivar. It originated in Tuscany, but it is now largely present in other regions of the country, often with different names (synonyms). It adapts to the most varied climates and, for this reason (and also for the high quality of its oil), it has been adopted in emerging olive-growing countries, such as the United States, Australia, South Africa, Argentina and Chile. It has an elongated fruit with 17–20% of oil content. 'Leccino' is another important Italian oil cultivar, originated from central Italy. The cultivar has a good resistance to strong frosts, which in several areas of Central Italy are periodically the cause of high damage to olive trees. Hence the cultivar is largely used in breeding programmes with the aim to select cold-resistant olive genotypes. Its elliptic-shaped fruit has around 17% oil content. 'Pendolino' is another cultivar largely used in olive orchards of central Italy, mainly because it is considered a good pollinator. 'Ogliarola' is the name of a group of cultivars of southern Italy characterised by high productivity and oil content. Other important Italian oil cultivars are 'Coratina', 'Canino', 'Carolea', 'Moraiolo' and 'Biancolilla'. Although Italy is one of the most important producers of olive oil, it is not considered among the leading countries for table olive production. Nevertheless, it has large-fruited cultivars, producing high-quality table olives. Among them, the cv Nocellara del Belice, which is similar to the Spanish 'Manzanilla' and the Greek 'Amphisis', is considered to be the best Italian table olive cultivar. 'Ascolana tenera' is another very important Italian cultivar, today

diffused also in other countries such as Israel, Mexico, Argentina and California. Among the dual-purpose cultivars, ‘Itrana’, ‘Giarraffa’ and ‘Tonda Iblea’ are worthy of mention.

‘Koroneiki’ is the main oil variety of Greece and is planted in over 50% of the country’s olive area, particularly in the Peloponnesus, in Crete and in other islands. It has a small ovoid-shaped fruit with high oil content; it is resistant to drought, but not to low temperatures. ‘Mastoidis’, ‘Kalamata’ (‘Kalamon’) and ‘Chalkidiki’ are other important Greek varieties with high oil content and a good flesh/stone ratio. ‘Amphisis’ (‘Konservolia’) is the main black table olive cultivar, constituting 80–85% of Greek table olive production. This cultivar is mainly grown in central Greece and it has a round-to-oval shaped fruits with the colour gradually changing, at maturity, from deep green to black with white dots.

‘Picholine’ is grown in Southern France and, together with the cultivar ‘Lucques’, is the most used in the French table olive industry. It is considered as a dual-purpose cultivar. It is also cultivated in Italy, Israel, Morocco and, occasionally, other olive-growing countries. Its fruit has 15–18% oil content under non-irrigated conditions. The oil is rather light in colour and of very high quality.

Turkey, Syria, Morocco and Tunisia, which have relatively high productions of table olives, generally prefer to use dual-purpose cultivars. Among them, Turkey and Syria have a high table olive consumption. ‘Ayvalik’ is the most important Turkish oil cultivar while ‘Domat’ and ‘Gemlik’ are those mainly used as table olives, where the former is consumed as green table olive and the latter is for black table olive (Ergulen et al. 2002). ‘Massabi’, ‘Sourani’ and ‘Temprani’ are among the best Syrian dual-purpose cultivars.

Other important cultivars are ‘Picholine marocaine’ and ‘Zitoun’ (Morocco), ‘Chemlali’ and ‘Chitoui’ (Tunisia), ‘Sigoise’ (Algeria) and ‘Nabali’. The latter, in particular, is a dual-purpose cultivar largely diffused in the Middle East, having a high oil content (about 30%).

For a detailed list of world olive cultivars, the ‘Olive Germplasm: Cultivars and World-Wide Collections’ database (http://apps3.fao.org/wiews/olive/oliv.jsp) can be consulted. The database is the 2005 web edition of a previous report (Bartolini et al. 1998) and contains information on 1,208 cultivars. Essential information on the main characteristics of cultivars (e.g., productivity, oil content and extraction, rooting ability, tolerance to abiotic and biotic stresses, biochemical and molecular identification) is also reported.

12.4 Olive Propagation

12.4.1 Traditional Propagation Techniques

In the olive-growing countries, olive propagation is achieved by rooting of leafy stem or softwood cuttings, by grafting pieces of stem (scions) onto seedlings or clonal rootstocks or, today only occasionally, by regenerating whole plants from the

ovules, that is, characteristic tissue hyperplasia that appear as protuberances at the collar of old trees (Fabbri et al. 2004). Among these techniques, rooting of leafy stem cuttings under mist is by far the most common technique. By the mid-1950s, the technique spread, especially in countries like Spain, where grafting had never acquired importance, and grew to become the source of over 70% of propagation material, leaving only about 20% of the market to grafted plants (Fabbri 2006). In general, cuttings are obtained from one-year-old or younger shoots by dividing them into 10–15 cm pieces of 4–6 mm in diameter, with 4–6 nodes and with the 4–6 leaves at the distal nodes maintained on the cuttings. In order to stimulate rooting, before insertion in the rooting substrate, the cuttings are treated at their basal ends with a root-promoting agent, that is, an hydro-alcoholic solution or a talcum powder formulation containing auxins, mainly indole-3-butyric acid (IBA), in high concentration (generally, 2,000–5,000 ppm). The basal ends of treated cuttings are then inserted 3–4 cm in the rooting medium (e.g., perlite), inside a rooting bench covered with a transparent plastic film, and maintained under mist conditions for the period necessary to form multiple and well-developed adventitious roots.

Several cultivars, mainly used as table olives, are very hard to root or do not root at all. In addition, the de-novo formed root apparatus is often poorly functional. Grafting is the only viable technique for clonal propagation of such cultivars. In comparison with cutting propagation, the production of grafted trees is a more complex operation that requires long practice and, as a consequence, is usually restricted to specialised nurseries where skilled labour is present. In olive, grafting is performed by inserting a small portion of a stem (scion) onto a clonal or a seed rootstock. Clonal rootstocks (i.e., rootstocks reproduced by cutting propagation) are used in Spain where they are obtained from specific olive cultivars (e.g., 'Verdal', 'Lechin de Sevilla', 'Oblonga' and 'Gordal').

The method of grafting scions on seedlings is still used in Italy and in some 'new' olive-growing countries, such as Argentina, where it has allowed a rapid diffusion of olive cultivation. In this technique, a short piece of shoot, mainly just one node, is grafted onto a rootstock that is developed from a seed. The main advantage of rootstock production by seed propagation lies mostly in the possibility of a cheap production of large numbers of high-quality virus-free rootstocks, even in nurseries having little skill and equipment. On the other hand, one drawback is that the seedlings are not homogeneous in terms of vigour and root development, hence influencing growth characteristics of grafted plants that can differ quite markedly. A proper handling of olive seeds (from fruit collection up to seed germination and seedling development) can greatly improve the characteristics of rootstocks, which in turn perform much better during grafting and contribute to produce well-developed grafted trees. In this sense, the use of high-quality seed (i.e., seeds of known provenance, clean and free from disease and insects containing viable embryos and showing high germinability) is of prime importance for rootstock production.

Several other techniques of propagation, based mainly on the traditions of ancient olive growers, have been developed in the time: (i) the use of rooted suckers or large cuttings from old branches (named 'estacas' and 'garrotes' in Spain), (ii) the

grafting on suckers or wild olive trees and (iii) the grafted-cuttings method (Fabbri et al. 2004). Few of them still maintain a certain importance in traditional areas of olive cultivation.

12.4.2 In Vitro Propagation (Micropropagation and Micrografting)

Micropropagation represents the most important advancement in plant propagation in the last 100 years. For a large number of species, a consistent improvement in the sanitary and qualitative characteristics of propagated plants was obtained after effective in vitro propagation protocols were developed. In plant breeding, micropropagation has become an important tool to reproduce large numbers of selected plants easily and in a shorter time, if compared to traditional propagation techniques. However, unlike the majority of fruit species, at the beginning of the 1990s only a few olive cultivars could be efficiently propagated in vitro by micropropagation (Rugini and Fedeli 1990). Moreover, at that time, micropropagation was often initiated using explants from embryos and seedlings (e.g., Bao et al. 1980; García-Berenguer and Durán González 1990; Cañas et al. 1992), but this approach is of minor interest when reproducing selected cultivars or clones. When using explants from adult trees, several problems hindered the development of effective protocols of micropropagation, among which (i) the heavy oxidation of tissues when explants (nodal segments and buds) are collected from in-field or greenhouse plants, (ii) the difficulty of getting sterile shoots when nodal explants were used and (iii) the laboriousness of establishing shoot cultures with some cultivars. Over the last decade, many advances have been made towards the solution of these problems and the optimisation of the various steps involved in olive micropropagation so that complete protocols (from the introduction in vitro of explants to the acclimatation of rooted plants) are today available for several cultivars from different Mediterranean countries (Lambardi and Rugini 2003; Giorgio et al. 2006).

12.4.2.1 Initiation and In Vitro Establishment of Shoot Cultures

The initiation of olive micropropagation using buds or nodal segments from adult field-grown plants is difficult and time consuming, mainly because of high contaminations and the rapid oxidation of tissues after plating. The same explants collected from potted stock plants, grown in greenhouse, are instead the ideal material to introduce in vitro the olive, particularly when tender apical twigs and nodal segments are excised from vigorous shoots soon after sprouting (Rugini and Fedeli 1990). Tissue disinfection before its introduction in vitro is a fundamental step in olive micropropagation, and the present tendency is to avoid the use of ethyl alcohol that causes tissue dehydration. Hence a treatment of 10–20 min with calcium or sodium hypochloride, at different concentrations, is the most common approach for explant disinfection (Mencuccini 1995).

In the micropropagation of olive, the development of a specific olive medium (OM) for shoot proliferation marked an important step towards the improvement

of the technique. The medium was formulated on the basis of the analysis of the main mineral elements of shoot apices from field plants during their rapid growth (Rugini 1984). The major differences between MS (Murashige and Skoog 1962) and OM medium formulations are: (i) the OM medium is richer in Ca, Mg, S, P, B, Cu and Zn, (ii) it has a slightly different Ca/N ratio (1:11) and (iii) it also contains glutamine as a nitrogen source. Unlike other fruit species, mannitol (one of the major carbohydrates of olive metabolism) has repeatedly proved to be the best carbon source in the shoot proliferation medium.

Olive is characterised by a strong apical dominance. As a consequence, shoot proliferation is achieved mainly by means of uni- or binodal segmentation of elongated shoots (Fig. 12.5, *top left*), instead of axillary bud proliferation – the typical approach with the majority of fruit species. Zeatin (a natural cytokinin) plays a major role in the regulation of this phenomenon. According to the olive cultivar, its concentration in the proliferation medium can range from 0.5 up to 10 mg/l.

Recently, micropropagation of olive in Temporary Immersion System (TIS) showed to be promising to limit the expression of shoot apical dominance and to increase proliferation rates (Lambardi et al. 2006a).

12.4.2.2 Shoot Rooting and Acclimatation

Great advances have been made in rooting of micropropagated shoots over the last decade so that even cultivars 'recalcitrant' to cutting propagation (such as several table olive cultivars) can now be satisfactorily rooted in vitro (Lambardi and Rugini 2003). The common approach in olive is to root elongated shoots when still in vitro by means of a simple transfer of single shoots to an auxin-containing medium. 1-Naphthaleneacetic acid (NAA) and IBA, at concentrations ranging from 1 to 4 mg/l, are generally used to root olive shoots. Over time, alternative or additional procedures to the traditional subculturing of shoots in a gelled auxin-containing medium have been proposed: (i) the 'pulse' treatment of shoots, that is, dipping for a short time the basal part of microcuttings in a highly concentrated auxin solution (e.g., Bartolini et al. 1990; Rugini and Fedeli 1990), (ii) the basal etiolation of shoots, performed by black painting of the outside of the jars and by covering the agarized rooting medium with sterile black polycarbonate granules (Rugini et al. 1993) and (iii) the addition of polyamines to the rooting medium (Rugini et al. 1997). However, these methods, although effective in enhancing in vitro adventitious rooting, never found practical application in olive micropropagation protocols.

Acclimation is another critical point in olive micropropagation due to the drastic change of climatic conditions (humidity, light intensity and asepsis) that characterises the passage from the in vitro to the in vivo environment. This problem is accentuated by the particular histology of leaves from in vitro culture, which makes them even more prone to desiccation, as well as poorly functional in the acquisition of autotrophic conditions. As a consequence, just after the exit from the in vitro conditions, olive plantlets are potted in small pots filled with appropriate compost substrates (e.g., peat moss, perlite and polystyrene granules, 2:2:1; Rugini and

Fedeli 1990) and acclimatated under a transparent plastic film or in fog conditions. Following acclimatation, a one-year hardening period is required before their final plantation in orchard (Mencuccini 1995).

To date, few reports have dealt with the genetic and agronomic characteristics of micropropagated olive trees after in-field plantation. However, no evidence has been produced as concerns the loss of the genetic fidelity of in vitro propagated trees to the donor plant (Garcia-Fèrriz et al. 2002; Leva et al. 2002). When transferred on to field, micropropagated plants have usually given satisfactory results with reference to overall growth and onset of flowering; moreover, the occasional appearance of juvenile traits is transitory (Briccoli Bati et al. 2002; Leva et al. 2002). Not all cultivars, though, respond equally well to micropropagation in terms of productivity (Briccoli Bati et al. 2006).

12.4.2.3 Micrografting of Olive

In addition to micropropagation, the micrografting technique has been recently proposed for the olive. Cycles of shoot micrografting on in vitro-grown seedling rootstocks, for instance, proved to be successful in inducing rejuvenation of mature olive trees (Revilla et al. 1996; Farahani et al. 2006). Moreover, Troncoso et al. (1999) cleft-micrografted uninodal explants (from in vitro-grown ‘Cañivano’ seedlings) on in vitro ‘Arbequina’ seedlings, prepared with a cut just under the basal pair of leaves, obtaining 67% of plantlet survival and hardening in vivo. These results suggest that micrografting should be further explored as an additional approach to olive multiplication.

12.5 Breeding Objectives

The recent diffusion of olive outside its traditional areas of growth, together with a continuous trend in the modernisation of its cultivation, has greatly increased in recent years the demand by olive growers of improved cultivars (e.g., more suitable to mechanisation and utilisation in intensive orchards). The large genetic variability of olive, as expressed by the high number of cultivar populations, could offer great opportunities for a marked improvement of olive characteristics. Notwithstanding, the genetic improvement of olive is still far from being comparable to that of other temperate fruit species. For a long time, old farmers, particularly in countries with ancient olive traditions, have considered the olive an ‘easy’ tree, which did not require the particular attentions (in terms of culture management and care) that were reserved for other fruit species, such as, for instance, the grape. Hence the onset of advanced studies for the improvement of olive culture is a relatively recent story. Indeed, an important impulse towards new research and development in olive culture and oil production came after 1974 from the FAO, which was the promoter of international projects based on modern scientific approaches. Soon the necessity of an effort to improve plant material was evident as traditional cultivars showed to be not always adequate to support the modernisation and the intensification of olive

orchards. In concomitance, although the Mediterranean basin is the area which still has 95% of the olive orchards of the world, over the last 30 years the production and the consumption of olive oil have greatly increased, particularly outside this elective area of cultivation, interesting countries of different Continents, such as Japan, China, South Africa, USA, Argentina and Australia. Remarkable increases in olive cultivation and oil production (up to 10 fold) have been observed in some of these countries, such as Australia. Hence, the volume of olive oil consumed annually worldwide is expected to soon exceed three million tonnes. Such volume of olive oil requires active farming programmes and selected olive trees for both new orchards and replacement in old olive groves. Moreover, as the olive industry moves from traditional manual methods to mechanised operations, planting stock will need to be developed to meet future challenges. As a consequence, selection is directed to genotypes that are early bearing, resistant to pests and to abiotic stresses (such as frost and drought), with a limited alternate bearing, suitable for intensive culture and mechanical harvesting and characterised by high-quality productions in terms of both organoleptic characteristics of fruits, and high content in substances useful for human health.

12.5.1 In-field Collections of Olive Cultivars

The collection, characterisation and preservation of olive cultivars can be considered the first fundamental steps against the risks of genetic erosion and towards the exploitation of genetic resources for breeding programmes. The renewal of old groves in the main olive-producing countries, the use of a limited number of cultivars more suitable for the new intensive and mechanised orchards and the diffusion of new cultivars, already available or which are going to be released by ongoing breeding programmes, are all factors producing a progressive abandonment of autochthonous and 'local' cultivars and, as a consequence, a real risk of erosion of olive genetic resources. In addition, a patrimony still exists of genetic resources to be characterised in traditional olive-growing countries outside Europe (e.g., in Tunisia, Morocco, Syria and Turkey) as well as a 'new-emerging' genetic variability in other countries (e.g., Argentina, California and Australia) due to the common use of seed propagation to produce rootstocks.

Because of that, in recent years various public institutions, both at the national and international level, have promoted a thorough conservation campaign with the goal to retrieve and preserve accessions from distant locations and countries where conservation is not provided. The most important olive international Institution, the International Olive Oil Council (IOOC), for instance, has been the promoter in 1995 of the European project RESGEN ('Conservation, characterization, collection and utilization of the olive genetic resources') aimed at the collection, characterisation and conservation of olive genetic resources as well as at the introduction of germplasm from different countries in national in-field collections. The project, financially supported by the European Union, was initially developed only for EU members (Spain, Greece, Italy, Portugal and

France), but soon the interest generated in other olive-growing countries opened the door to the participation of nine more IOOC Members, that is, Algeria, Syria, Morocco, Tunisia, Cyprus, Egypt, Israel, Slovenia and Croatia (Essid 2006). As a main result of RESGEN, more than 1,400 accessions have already been collected in national repositories, 500 of which are autochthonous cultivars (see http://www.internationaloliveoil.org/resgen/index.html). In these clonal orchards, the trees are maintained, agronomically evaluated, morphologically and molecularly described, and are the source of propagation material. In addition, an action aimed at the identification and the survey of forests of the Mediterranean basin, containing rare wild and feral forms of olive trees, is also ongoing (Ouazzani and Lumaret 2006).

Important collections are today present in all the main olive-growing countries. The most important is in Spain where the collection of the Olive World Germplasm Bank (OWGB) of Cordoba accounted in 2005 for more than 400 accessions from 20 olive-growing countries, about half of which are already registered and authenticated by means of morphological descriptors and/or molecular markers. The collection is continuously implemented with new accessions from Spain and other countries (Caballero et al. 2006). In Tunisia, a germplasm bank was established in 1990 by the National Conservatory of Boughrara-Sfax, and contains at present about 120 accessions comprising autochthonous varieties, local forms and foreign cultivars (Trigui et al. 2006). In Italy, in addition to various national and international clonal collections established by public institutions, a descriptor-list of Italian cultivars (from Tuscany) has been realised and is available for consultation in internet (Iannì et al. 1995, http://www.ivalsa.cnr.it/archivio%20fruit/olivo/indice.htm).

12.5.2 Main Characters for Olive Improvement

In the last two decades, various olive-growing countries (Spain, Italy, Israel and Greece) have been the promoters of programmes for olive improvement based mainly on the direct observation, clonal selection and cross-breeding of plants from local cultivars, exhibiting interesting phenotypic characteristics. Two main reasons can explain this traditional approach to the improvement of olive genetic resources: (i) local cultivars are the result of a ‘natural’ selection, that is, the process of adaptation of a plant to a specific environment in order to optimise its growth and functions. In terms of productivity (quantity and quality of olive products), the process was driven by the hand and the expertise of old olive growers. Local cultivar populations are the result of this long-lasting process, and they present satisfactory characteristics of productivity and adaptability to the climatic and pedological conditions of the specific area of cultivation. The main drawback lies in the fact that, when moved to a different environment, out of their area of origin, these cultivars often show great difficulties of adaptation with a consequent decrease of their yield performance; (ii) in olive, almost nil is the information available concerning the genetic control of characters and their hereditability.

Olive improvement is therefore at present focussing its attention on the evaluation of adaptability of the main cultivars to different areas of cultivation, through the establishment of clonal collections where genetic resources from different countries are not only preserved, but also comparatively evaluated. Specific programmes of clonal selection and cross breeding are ongoing, mainly at national levels, including quite a high number of agronomic and production characters. In addition, due to the maintenance of some traditional peculiarities in each olive-growing country, minor characters that are of interest in one country can be almost neglected in others. For instance, the characters 'germinability of seeds' and 'seedling morphology' are considered important only in Italy for the improvement of olive rootstocks as this country still has an economically important nursery production of plants grafted onto seed rootstocks (Fabbri et al. 2004). A list of the characters at present considered of major importance in olive breeding is reported in Table 12.2.

In recent years, several olive breeders concentrated their attention to the 'vigour and form' of the plants in order to select compact genotypes suitable for the new intensive orchards. Vigour and form are characters strictly linked in olive, and some methods were proposed in the past for a precocious characterisation of low-vigour genotypes, among which are the content in abscisic acid in shoots and leaves (Yadava and Dayton 1972) and stoma density in leaves (Bartolini et al. 1979). However, Del Rio et al. (2002) reported that the characterisation of young plants with reference to their high or low vigour cannot start before they are at least 6-years old. Initially the breeding activity concerning dwarf plants produced interesting results in terms of new cultivars suitable for ornamental purpose (Hartmann 1967; Roselli and Donini 1982). Then the interest moved to the selection of compact cultivars (such as one selection from the cv Frantoio, the 'Fs-17'; Fontanazza et al. 1990) as well as dwarfing rootstocks (Buffa et al. 2006) with the aim to promote high-planting density and mechanisation of pruning and harvesting operations.

As regards fruiting and fruit characteristics, plenty of efforts have been put in the selection of cultivars with high or low oil content and, more recently, of dual-purpose cultivars. Investigations in this field had already hypothesized a genetic control of the oil content in fruits, a character showing high variability among the different cultivars (Fontanazza and Bartolozzi 1998). Unlike oil cultivars, requirements for table olives are low oil content to favour preservation and high reducing sugar contents to ensure a good lactic fermentation. A cultivar with these characteristics, 'Kadesh', was selected by Lavee (1978) and became fairly popular in Israel and Argentina.

Another character worthy of mention for its economical consequences is alternate bearing. This is a widespread phenomenon in olive cultivars, with negative effects on fruiting, vegetative growth and, as a consequence, on tree management (Lavee 2006). To limit the expression of alternate bearing, particular attention is required during fertilisation and pruning of olive trees, which in turn cause an increase of costs for skilled hand labour. Significant differences have been observed in the leaf protein content in fruiting and non-fruiting trees as well as in its quantitative change during the growing season (Lavee and Avidan 1994), supporting the hypothesis of specific gene activation or repression. Research in this topic is moving towards the characterisation of genes involved in flower bud induction.

Table 12.2 Examples of studies on characters important for olive improvement and relative cultivar selection

Character	Aim of investigation	Selected Cultivar	Reference
Tree vigour and form	Selection of dwarf forms, suitable for ornamental scope	'Swan Hill'	Hartmann (1967)
		'Briscola'	Roselli and Donini (1982)
	Selection of dwarfing rootstocks	'Fs-17'	Fontanazza et al. (1990)
			Buffa et al. (2006)
	Studies to find a correlation between physiological and morphological characters of young trees and their vigour		Bartolini et al. (1979)
			Del Rio et al. (2002)
Fruiting and fruit characteristics	Studies on alternate bearing		Lavee and Avidan (1994) and Lavee (2006)
	Short juvenility (early flowering) of trees		Leitão (1990), Bellini (1993) and Santos Antunes et al. (1999)
	Oil yield		Fontanazza and Bartolozzi (1998)
	Selection of cultivars with high-oil content	'Barnea'	Lavee et al. (1986)
	Selection of cultivars with low-oil content (table olives)	'Kadesh'	Lavee (1978)
	Selection of dual purpose (table olives and oil) cultivars	'Arno', 'Tevere', 'Basento'	Bellini et al. (2002)
	Selection of clones of 'Chemlali Sfax' for quality and regularity of production		Grati Kamoun et al. (2002)
	Hereditability of fruit characters in table olives		Fanizza (1982)
	Hereditability of fruit characters (size, weight and colour) in progenies from oil cultivars		Bellini (1993) and Parlati et al. (1994)
	Genetic variability of fruit histological characteristics		Mulas (1994)

Table 12.2 (continued)

Character	Aim of investigation	Selected Cultivar	Reference
Adventitious rooting	Improvement of rooting efficiency by means of *Agrobacterium rhizogenes* transformation		Rugini and Mariotti (1992), Rugini (1992), and Rugini et al. (2000)
	Progeny evaluation as regards rooting potential of cuttings		Voyiatzi et al. (2002)
	Classification of olive cultivars with reference to high, medium and low rooting ability		Fabbri et al. (2004)
Resistance/susceptibility to pests	Selection of cultivars resistant to *Spilocaea oleagina*	'Maalot'	Lavee et al. (1999)
	Selection of cultivars resistant to *Verticillum* wilt	'Oblonga'	Hartmann et al. (1971)
	Selection of cultivars resistant to *Cycloconium*		Lavee (1990)
	Classification of cultivars as resistant, tolerant and susceptible to the main olive phytopathologies		Bellini et al. (2003)
Tolerance to cold and frost conditions	Variability among Italian cultivars to frosts		Antognozzi et al. (1994, La Porta et al. (1994) and Bartolozzi and Fontanazza (1999)
	Studies on correlations between morphological/physiological characters and cold tolerance		Roselli et al. (1989), Roselli and Venora (1990) and Bartolini et al. (1999)
Salt tolerance	Studies on physiological and anatomical characters of salt tolerant plants		Gucci and Tattini (1997), Cantos et al. (2002)
	Different salt tolerance of olive cultivars		Therios and Misopolinos (1988), Tattini et al. (1994), Benlloch et al. (1994), Marin et al. (1995) and Chartzoulakis et al. (2006)
	Selection in vitro of salt tolerant genotypes		Fodale et al. (2006)

The improvement of adventitious rooting ability of olive cultivars is a character of high interest for the nurserymen. The character is under genetic control, as proved by the very high variability of rooting potential (both natural and after auxin treatments) when cuttings are collected from different cultivars (Fabbri et al. 2004). Here, promising advances have been made using a biotechnological approach (see Section 12.7.4).

Up-to-date, specific knowledge on the genetic control of the mechanisms of resistance/susceptibility of cultivars to biotic stresses is still lacking. Notwithstanding, generic information on different levels of resistance of cultivars to the main pests affecting the olive tree is available (Bellini et al. 2003), and on these bases important results have been obtained in time with the clonal selection of genotypes resistant to the peacock leaf spot (*Spilocaea oleagina*; Lavee et al. 1999) and the *Verticillium* wilt (*Verticillium dahliae*; Hartmann et al. 1971). It is still difficult, on the other hand, for the selection of genotypes resistant to the olive fruit fly (*Bactrocera oleae*) and to the olive moth (*Prays oleae*), among the most dangerous insects in olive because of their severe damage to fruits and, in the case of olive moth, to flowers and leaves. Indeed, up to now, it has not been possible to find cultivars showing clear evidence of resistance or tolerance to these pests, which are common in several olive-growing areas where they are often the cause of great losses of product and marked decrease of oil quality.

Also the studies concerning tolerance or susceptibility of olive cultivars to abiotic stresses (mainly frost and salt) are of great interest and economical importance. Frost is one of the main problems in olive-growing areas where winter temperatures fall frequently to 10°C or more below zero. This condition is typical, for instance, of central Italy and, together with early and late frosts (in spring and fall), has repeatedly been in the past the cause of great losses of olive groves due to the death of the epigeic part of the trees. Hence, particularly in Italy, research has moved towards the characterisation of cultivars in terms of tolerance to low temperatures. Information is today available on the fair tolerance to winter frosts of some important cultivars, such as ‘Leccino’, ‘Ogliarola’, ‘Itrana’, ‘Tanche’ and ‘Moresca’; on the contrary, ‘Frantoio’ and ‘Moraiolo’ are highly susceptible (e.g., Antognozzi et al. 1994; La Porta et al. 1994; Bartolozzi and Fontanazza 1999). Some authors also report of an intra-cultivar variability of the cv Leccino to low-temperature tolerance (La Porta et al. 1994; Bartolini et al. 1999).

It has been repeatedly reported that the osmotic stress has several consequences on the vegetative growth of trees, particularly of the epigeic part. When growing in soils with high salt concentrations, the shoots have short internodes, small and thick leaves, and fruits of smaller size. The tree shows a general appearance of stunted growth and its productivity (both in terms of quality and quantity) can be negatively affected (Cresti et al. 1994). Several studies focussed attention on the evaluation of salt tolerance of different cultivars, assuming that, in general, ‘susceptible’ cultivars do not tolerate over 20 mM NaCl in the soil circulating solution, while ‘tolerant’ cultivars can resist up to 100 mM (Gucci et al. 1995). However, among the morphological symptoms that can be taken into consideration as ‘markers’ of salt tolerance/susceptibility, the quantification of growth reduction of trees

under osmotic conditions seems to be the most promising. Based on that, some of the main world cultivars are considered 'tolerant', such as the Spanish 'Arbequina', 'Lechin de Sevilla' and 'Picual' (Benlloch et al. 1994), the Italian 'Frantoio' (Tattini et al. 1994), the Tunisian 'Chemlali' (Ben Ahmed et al. 2006) and the Greek 'Megaritiki' (Therios and Misopolinos 1988), 'Kerkiras' and 'Kalamata' (Chartzoulakis et al. 2006).

12.6 Breeding Techniques

12.6.1 Clonal Selection

Due to the occurrence of self-incompatibility in olive germplasm, the cultivar populations of olive have a high degree of heterozygosity and the genetic variability is consequently high. This means that the potential for improvement is relevant, and by no doubt this feature of the olive has made possible the agronomical evolution of the crop in the Millennia, also because olive suitability to agamic propagation enabled farmers to preserve the selected types by vegetative propagation. As a result, the main producing countries of the Mediterranean basin possess hundreds of major and minor cultivars that represent an unfathomed variability which might yield the characters most useful for modern olive industry.

Clonal selection was proposed in the early 1960s for olive improvement in order to avoid the problems and the long time required for the development of programmes of cross-breeding and selection. Today, though considered a slow breeding technique, it still remains a valuable instrument that has been employed also recently in a number of olive producing countries, making it possible the improvement of the standard of numerous cultivars, as well as the increase of their homogeneity in terms of agronomic and productive characters. An additional positive aspect of clonal selection is the sanitary control that usually accompanies the procedure: the lines emerging from the clonal selection procedure are, as a rule, virus-free and more tolerant to pathogens, thus contributing to a general improvement of the industry.

In the last 50 years, in spite of the large amount of resources needed to pursue this kind of genetic improvement, all olive producing countries have promoted clonal selection programmes with often encouraging results (Lavee 1990). Selections were particularly initiated in regions with large-scale autochthonous olive populations and continued under different growing conditions, climates and levels of intensification (Lavee and Avidan 2002). Clones of standard cultivars have already been selected or are under evaluation in many countries, such as:

Spain, where the first studies were aimed at the improvement of cvs Picual, Manzanillo and Hojiblanca for characters of productivity and for the reduction of alternate bearing (García-Berenguer 1978). Later on, attention of breeders moved mainly to 'Manzanilla' (Suárez, Lopez-Rivares et al. 1990) and 'Arbequina' (Tous et al. 1993), and both these works resulted in the first selection of many interesting clones. One of them, the 'Arbequina' clone 'I-18', has had commercial diffusion for

its improved characteristics of productivity and the upright form of growth, which makes it suitable for mechanical harvesting;

Italy, working initially for the improvement of the cvs Frantoio, Moraiolo and Leccino, in order to improve their characters of productivity and winter-hardiness (Morettini 1961; Bartolini et al. 1995). An interesting clone of unknown origin, the 'I-77', was selected by Fontanazza (1993), having interesting characteristics of low vigour, self-fertility and early onset of bearing. At present, clonal selection is more active in the south of Italy and promising clones have already been obtained for the cvs Carolea (Parlati et al. 1995), Tonda Dolce (Mulè et al. 1992) and Nocellara del Belice (Mulè et al. 1994).

Portugal, where, as a result of a long-lasting clonal and sanitary selection programme, concerning 10 cultivars from the southern part of the country, 27 clones were selected, established in the field and are now under evaluation, mainly for characters of earliness of flowering and fructification (Serrano et al. 1999);

Tunisia, where the international restrictions on the local olive oil due to its fatty composition (i.e., oil rich in saturated fatty acids and poor in oleic acid) have imposed an improvement of local cultivars. In particular, an important programme of clonal selection is in progress with the cv Chemlali Sfax, aimed at the selection of genotypes more productive and able to give the quality of oil that can meet the international market criteria (Grati Kamoun et al. 2002).

Programmes of clonal selection have also been started in other countries, such as in France (e.g., with the cvs Picholine, Tanche and Lucques), Morocco (with the cv Picholine Marocaine), Turkey (mainly with the cvs Memecik, Ayvalik and Gemlik), Israel (with the cvs Souri and Nabali) and Cyprus (with the cv Local) (Bellini et al. 2003).

12.6.2 Cross Breeding

Cross breeding in the olive is usually more difficult and time consuming than in other fruit tree species. A main reason for this difficulty is the relatively scarce knowledge of the hereditary behaviour of the most important bioagronomic traits. Then additional objective obstacles are: (i) the high heterozygosity of the species (Rugini and Pannelli 1993) and its high degree of polymorphism; (ii) the self- and cross-incompatibility characteristics typical of the species, a condition which limits or makes harder the attempts at self-crossing or inter-crossing the cultivars; (iii) the long-lasting juvenile phase of plants, which is usually in the 10-year range or more. This, in turn, means that a minimum time for the release of a new cultivar is 20 years or more; (iv) the difficulty at emasculating the flowers, with the consequence that the nature of the pollen and of the parent tree can at times be uncertain. Also because, even in the case of self-incompatibility, a minimum amount of self-fertilisation can occur; and (v) the low fruit set rate (Rugini and Lavee 1992), and the even lower number of mature fruits containing vital seeds.

In addition, the linked heredity of various olive characters has to be taken into careful consideration in breeding activities, as in the classical example of the inverse

correlation between the oil content and the fruit size, which can be regarded as 'negative' for oil cultivars and 'positive' for the table olive ones.

As a consequence of all these problems, the olive has not received in recent decades the attention scientists gave to other fruit crops as concerns genetic improvement using cross-breeding approaches. This fact is clearly evidenced by the very limited number of new cultivars and rootstocks that have been released in the last 30 years (see Table 12.2). However, a promising change in this trend has been observed, as advances in the breeding technique (e.g., the in vitro embryo culture allowing the germination of naked embryos and the methods to shorten the juvenile period of plants) enhanced in recent years the onset of cross-breeding programmes in several olive-growing countries. Some of these programmes have already produced the first promising results (Fig. 12.2). As for the clonal selection, the majority of these programmes are today aimed at improving the autochthonous cultivars, and breeding activities are mainly based on selection within the F1 progenies, which display a marked variability even when self-pollination is adopted. Further improvement of cultivars for specific characters requires the utilisation of

Fig. 12.2 'Basento', a new olive cultivar released in the frame of a programme started in Italy in 1971. It is a dual-purpose olive cultivar obtained by cross-breeding ('Picholine′ × 'Manzanilla′) with semi-compact habit and high fertility. The characteristics of the fruit are good size, a very high flesh/stone ratio (> 19), medium-low oil content (11%) and very good organoleptic quality (Bellini et al. 2002)

second (F2) and third (F3) generation progenies. Hence the inclusion of characters from more than two parents and back crosses to amplify or reduce specific characters are presently a common strategy for olive genetic improvement.

12.6.3 Mutagenesis

Studies on induced mutation were first carried out in Italy by Morettini (1954b), in an attempt to find a solution to the problems related to the long juvenile phase of olive plants in breeding programmes. Later, advances were due to the studies of Donini, who applied X-rays (1975) and γ-rays (1976) to induce genetic alterations. Roselli and Donini (1982) were the first to patent a new cultivar, the cv Briscola, obtained by irradiation of self-rooted plantlets of 'Ascolana Tenera' (Fig. 12.3). Shoots of 'Briscola' have short internodes, a character that confers the plant a general dwarf form, resulting very interesting for ornamental purposes (Fig. 12.4).

By irradiation of 'Frantoio' and 'Leccino' self-rooted plants, also Pannelli et al. (1990) obtained two vigorous but compact mutants (one for each cultivar) as well as one dwarf 'Leccino' mutant. The plants showed several morphological and physiological differences from the mother plants, such as shorter internodes, larger and thicker leaves, higher assimilation rates and water stress tolerance. Moreover, in vivo and in vitro selection allowed the isolation of tri- and tetraploid plants obtained

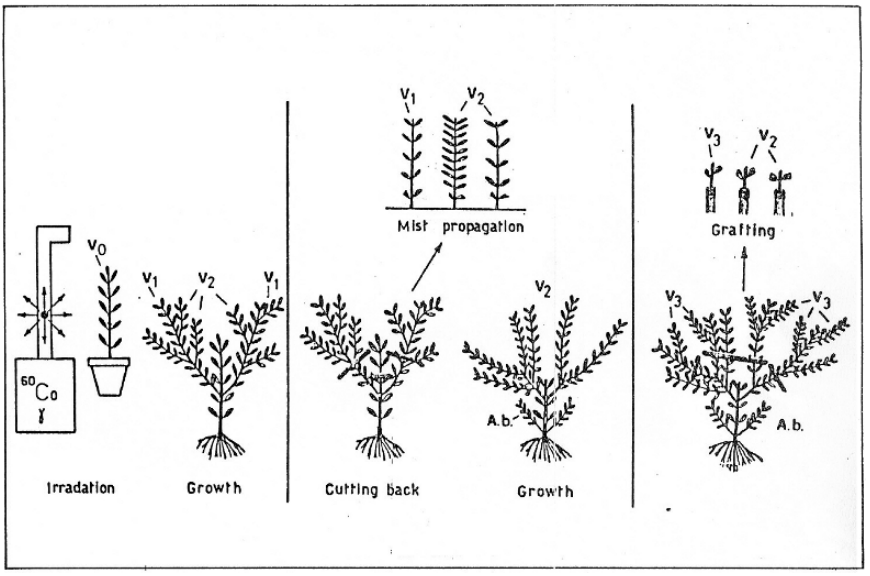

Fig. 12.3 Scheme proposed by Scaramuzzi and Roselli (1986) for the isolation of somatic mutations in olive trees, originated following the exposure of self-rooted plants to γ-rays. As the mutants always assumed chimeric forms, some cycles of cutting and grafting propagation were necessary to select wholly mutated shoots

Fig. 12.4 'Briscola' is an ornamental form of olive, which was obtained by induced mutagenesis, according to the scheme of Fig. 12.3. The cultivar is characterised by short internodes (*top*) and slow growth, which confer a general dwarf form (*bottom*) and make the tree particularly attractive for ornamental purposes (photos courtesy of G. Roselli and the 'SPO', Società Pesciatina di Orticoltura of Pescia, Italy)

by axillary bud stimulation of mixoploid mutants. In this way, plants were selected with a prevalence of tetraploid cells, which, by virtue of a thicker cell wall, showed to be more resistant to the peacock eye (Rugini et al. 1996). This study is still in progress to test the use of triploids and tetraploids plants as rootstocks (Rugini et al. 2006).

12.7 Biotechnological Approaches to Olive Improvement

In the last two decades, procedures of in vitro regeneration other than traditional micropropagation have been developed in olive, such as somatic embryogenesis and organogenesis from callus culture and the synthetic seed technology. The main

reason was to explore non-conventional methods of plant propagation, germplasm conservation and genetic improvement. Somatic embryogenesis, in particular, has been largely investigated because (i) it can be employed for mass micropropagation of plants, also overcoming difficulties faced in rooting numerous olive cultivars, (ii) dihaploid homozygous plants can be obtained from reproductive organs, such as anthers, pollen or ovules (Perri et al. 1994; Rugini et al. 1995), (iii) it can be used for the production of synthetic seeds (see Section 12.7.3), (iv) it can produce new variability via somaclonal variation and genetic transformation using either *Agrobacterium* or microprojectile bombardment techniques (see Section 12.7.4) and (v) it can be used in cryopreservation, providing an additional and powerful tool for the safe preservation of olive germplasm (see Section 12.7.5).

12.7.1 Somatic Embryogenesis

12.7.1.1 Induction of Embryogenic Lines from Zygotic Embryo and Seedling Explants

In olive, somatic embryogenesis has been induced mainly from juvenile explants, that is, immature (Rugini 1988; Leva et al. 1995) or mature zygotic embryos (Orinos and Mitrakos 1991; Mitrakos et al. 1992) with or without callus interposition. These studies showed that the maturation degree of the original explant has particular importance for the induction of somatic embryogenesis, either when entire zygotic embryos or excised cotyledonary explants are used. In the former system, somatic embryogenesis was reported only when zygotic embryos were harvested 75 days after full bloom and cultured in half-strength MS medium containing 0.5–2.5 μM BA (Rugini 1988). The existence of a 'window' of embryogenic competence during zygotic embryo development was also reported by Leva et al. (1995). They observed that only cotyledonary explants from immature embryos (cvs Picholine, Frangivento and Frantoio), harvested between 60 and 90 days after anthesis, were competent for embryogenic callus induction following their culture in SH (Schenk and Hildebrandt 1972) medium containing various combinations of NAA and isopentenyladenine (2iP). When cotyledons came from earlier (30 days) or later (130 days) collections, no evidence of somatic embryogenesis was observed. Differently, embryo-like structures could be observed in calli, originated from cotyledonary explants, when 'Chalkidikis' zygotic embryos were harvested 126 days after full bloom (Pritsa and Voyiatzis 1999).

A dissimilar morphogenetic expression of calli from different zygotic embryo tissues has also been evidenced in olive. Indeed, when mature embryos of the cv Koroneiki were used as the source of explants (Mitrakos et al. 1992), both rhizogenesis and somatic embryogenesis were high from radicle calli and low from distal-cotyledon calli, while only high rhizogenesis was promoted in calli from proximal cotyledon segments. It is notable that the high level of somatic embryogenesis (up to 40%) was obtained in radicle calli that, after 14–21 days in induction medium, were subcultured on OM medium without exogenous growth regulators. The high

embryogenic potential of root callus was confirmed in trials with seedlings of the cvs S. Agostino (Rugini et al. 1995) and Nabali (Shibli et al. 2001).

12.7.1.2 Induction of Embryogenic Lines from Mature Tissue Explants

Induction of embryogenic callus lines from mature tissues is by far the most useful technique for application to transformation studies of trees. However, in olive this approach was proved to be very difficult as, up to now, only one report is available where an effective embryogenic line was obtained from mature tissues (leaf petioles) excised from the cvs Canino and Moraiolo (Rugini and Caricato 1995). The regeneration system is described better as a 'secondary somatic embryogenesis' due to the fact that, once the embryogenic line was established, cycles of secondary somatic embryos were obtained directly from the epidermal tissue of primary somatic embryos (Fig. 12.5, *top right*). This way, the embryogenic line can be maintained for years by monthly subculturing. Histological observations showed that, in the embryogenic masses, together with a majority of perfect somatic embryos, sev-

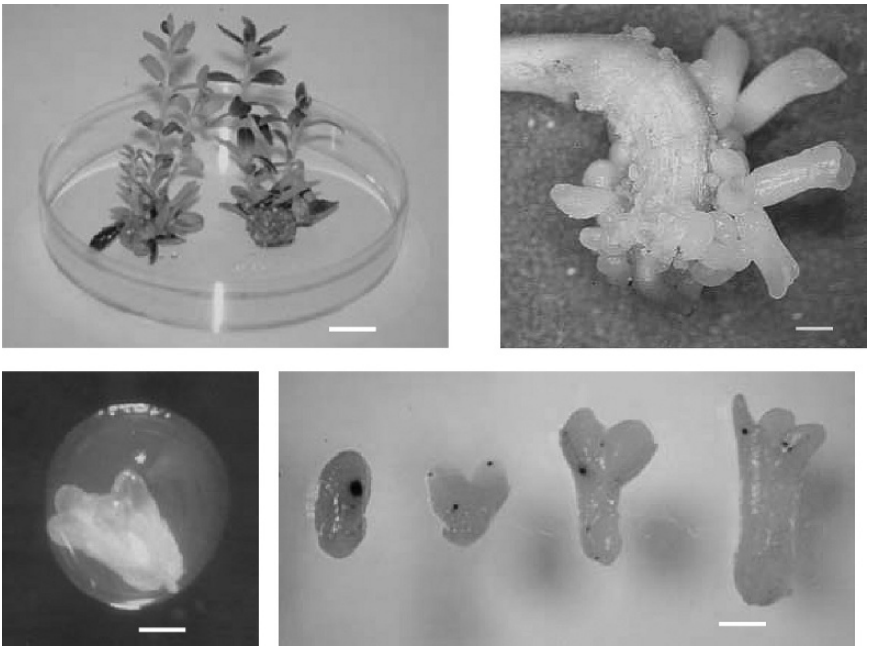

Fig. 12.5 Application of biotechnologies to the propagation and the genetic improvement of olive. *Top left*, Micropropagated shoots of the cv Frantoio just before to be cut at their base and transferred to the rooting medium (bar, 1 cm). *Top right*, Somatic embryogenesis in olive: many secondary somatic embryos, formed at the radicle end of a primary somatic embryo, with no evidence of interposed callus (bar, 1 mm). *Bottom left*, A synthetic seed of olive, containing a somatic embryo (bar, 1 mm). *Bottom right*, GUS gene expression in somatic embryos of olive at different stages of development, following their microprojectile bombardment (bar, 1 mm) (photos from Lambardi and Rugini 2003; *bottom right*, original photo of M. Lambardi)

eral other forms are generated, such as fused embryos, teratomic leaves, claviform structures and embryos with fused cotyledons (Benelli et al. 2001a).

12.7.2 Shoot Organogenesis

Since adventitious shoots and roots were obtained for the first time from seedling explants (Gilad and Lavee 1974), few reports have dealt with olive regeneration by shoot organogenesis. Direct shoot regeneration was induced on olive hypocotyls in White medium (White 1939), supplemented with 0.05 μM NAA and 2.5 μM BA (Bao et al. 1980) as well as from cotyledons of mature seeds (Rugini 1986). High shoot organogenesis was obtained from callus, previously induced on cotyledon segments excised from 'Tanche' and 'Picual' embryos (Cañas and Benbadis 1988). Initial callus proliferation was produced on OM medium containing high auxin/cytokinin ratio. Shoot organogenesis was then stimulated when the calli were transferred onto a 2iP-containing medium. Maximum shoot regeneration was observed in calli induced from cotyledon segments proximal to the embryo axes rather than the distal ones, suggesting that a gradient of regeneration potential existed from the proximal to the distal region of olive cotyledons. Rooting of adventitious shoots was obtained by transferring them to an IBA- or NAA-containing OM medium.

Mencuccini and Rugini (1993) obtained adventitious buds in petioles from in vitro-grown shoots of olive (cvs Moraiolo, Dolce Agogia and Chalkidikis). Interesting findings of this study concerned the efficiency of shoot organogenesis, which were heavily dependent on: (i) the cultivar ('Moraiolo' being the best), (ii) the position of the petiole along the shoot (apical nodes being better than basal ones), (iii) the medium/hormone combination and (iv) the culture in dark condition. Following rooting, the regenerated plantlets did not show morphological differences in comparison with the micropropagated donor plantlets.

12.7.3 Synthetic Seed Technology

Synthetic or artificial seeds (also named 'synseeds') are a recent evolution of tissue culture aimed not only at improving conventional micropropagation, but also at the easy storage of plant germplasm. Not only has the technology been mainly developed with ornamental species (Lambardi et al. 2006b), but also explants from fruit species can be successfully used for the production of synseeds (Standardi and Piccioni 1998). The synthetic seeds can be defined as 'artificially encapsulated somatic embryos, shoots, or other tissues which can be used for sowing under *in vitro* or *ex vitro* conditions' (Aitken-Christie et al. 1995). Synthetic seeds have also been recently tested for olive (Fig. 12.5, *bottom left*) with promising results. Micheli et al. (2002) used both microcuttings (apical and axillary buds) and somatic embryos of olive to produce synthetic seeds. The explants were first immersed in sodium

alginate solution (2.5%), after which drops of the solution (each drop containing one explant) were released into a complexing solution, that is, a water solution containing 100 mM of $CaCl_2$. The hardened beads were then washed from the solution and stored; after 'sowing' on an appropriate medium, they germinated successfully and converted into plantlets. Moreover, rooting and conversion to plantlets of 'Moraiolo' nodal segments are improved by dipping synthetic seeds in a sucrose- and IBA-containing solution before sowing (Micheli et al. 2006).

12.7.4 Genetic Transformation of Olive

The application to olive of genetic transformation studies dates back to 1984, when Rugini simply inoculated *A. rhizogenes* to the middle (by puncture) or the base (by longitudinal wounding) of in vitro-grown shoots of 'Dolce Agogia', with the aim of increasing its rooting potential by means of the creation of chimeric plants (Rugini 1986). A few years later, it was reported that the culturing of *A. rhizogenes*-inoculated shoots in putrescine-containing media dramatically increased rooting rates and basal callus formation (Rugini 1992). In spite of these first promising results, eventually olive was not intensively involved in genetic transformation studies mainly due to the difficulty of obtaining efficient morphogenetic lines from mature tissue that, once transformed, can guarantee high rates of regenerative events. In addition, the major olive-growing countries were subjected to the EU *moratorium* against 'Genetically Modified Organisms' (GMOs) and this condition is still a major obstacle to get significant advances in this research area. Notwithstanding, scientists are convinced that the genetic transformation of olive can be an important alternative to traditional breeding able to speed up the development of new genotypes improved for specific characters. For an exhaustive review of this topic, the reader is addressed to Rugini et al. (2000).

12.7.4.1 Transformation Techniques

Evidence has been produced showing the possibility to insert foreign genes into olive cells through both indirect (via *Agrobacterium*) and direct (by means of the biolistic technique) DNA transfer. *Agrobacterium*-mediated transformation was mainly used with *rol* genes of *A. rhizogenes*, in order to increase the potential of olive to produce adventitious roots. With this aim, suitable wild types of *A. rhizogenes* were used to isolate *rol* ABC genes. Then these genes, contained in p1855 and pBR322 plasmids, were cloned in LBA4404 strain of *A. tumefaciens* to transform olive tissues (Rugini et al., 2000). Selectable markers, allowing to distinguish transformed tissues by preventing the growth of the untransformed ones, play a crucial role for the development of transformation procedures. In olive, efficient selection of transformed cells was achieved by using the antibiotic kanamycin (50–100 μg/ml, depending on the type of explant) after 3–4 weeks of co-cultivation of explants with *Agrobacterium*, in order to increase the number of transgenic cell colonies (Rugini et al. 2000).

Besides the *Agrobacterium*-mediated technique, olive has been tested also for direct gene transfer by means of the microprojectile-DNA delivery system. The technique is based on the direct release into cells of specific genes (inserted into plasmid vectors, in turn adsorbed on the surface of gold or tungsten particles) by means of particular devices working at high helium pressures. In olive, the technique was applied to study transient gene expression of somatic embryos (cv Canino), following the optimisation of delivery parameters such as the pressure of bombardment, the type of particles (tungsten or gold) and the kind of particle delivery device, that is, the Particle Inflow Gun (PIG) or the Particle Delivery System (PDS)-1000/He (Lambardi et al. 1999). In this study, different plasmid vectors (the pZ085, containing the 35S promoter fused to the GUS gene, and the pCGUδ0, containing the sunflower ubiquitin promoter fused to the GUS gene with ubiquitin intron; Binet et al. 1991) were used to bombard somatic embryos at different stages of development. GUS gene expression could be detected in somatic embryos that were bombarded with gold particles (Fig. 12.5, *bottom right*), provided that appropriate delivery pressures were optimised for both devices. More recently, the biolistic system has been used also for the transformation of embryogenic cultures from 'Picual' juvenile material, using three different plasmids, that is, the pGUSINT (containing the 35S promoter), the pJGUS5 (in which the 35S promoter is coupled to an enhancer of expression) and the pCGUδI (Pliego-Alfero et al. 2005).

As mentioned, transient gene expression has been tested in olive following microprojectile DNA-delivery on somatic embryos of 'Canino' (Lambardi et al. 1999) and 'Picual' (Pliego-Alfero et al. 2005). It is interesting to note that, with the application of the β-glucuronidase histological assay, both reports evidenced the highest GUS gene expression when the pCGUδ0 plasmid, containing the ubiquitin promoter, was used for transient transformation.

12.7.4.2 First Attempts to Transfer Specific Genes in Olive

Rol genes of *A. rhizogenes* have been largely investigated with the aim to increase the potential of adventitious rooting of olive cultivars. In the abovementioned pioneer study of Rugini (1984), although the roots emerging from inoculated shoots were rarely transformed, an increased rooting ability with more secondary roots was observed, possibly demonstrating the inductive role of partial integration of T-DNA on the non-transformed neighbour cells (Rugini and Mariotti 1992). Afterwards, transformation procedures have been developed to transfer *rol* ABC genes to zygotic immature embryos of 'Moraiolo' (Rugini and Fedeli 1990) and to leaf petioles of 'Dolce Agogia' and 'Moraiolo' (Mencuccini et al. 1999) with promising results. The same gene construct (i.e., LBA 4404 strain of *A. tumefaciens*, encompassing the *rol* ABC genes in pBIN19 plasmid and the *nptII* gene encoding resistance to the kanamycin) was used in these studies. Selected plantlets, originated from transformed embryogenic calli, showed short internodes and high root potential. The transgenic plants are now under evaluation in experimental fields in Italy (Rugini et al. 2006).

Another interesting research line was developed to increase fungal disease resistance in olive. With this aim, somatic embryos of the cv Canino were transformed with the *A. tumefaciens* strain LBA 4404, containing the *osmotin* gene under the control of the 35S promoter. The somatic embryos, after selection for transgenicity, originated osmotin plant clones with no sign of phenotypic alterations (Rugini et al. 2000), but with promising characteristics of resistance to the peacock disease (Rugini et al. 2006).

It is expected that, in the near future, olive genetic transformation will be oriented mainly to induce changes in the tree morphology, for example, to produce dwarf and semi-dwarf plants with a large and well-developed root apparatus, characteristics that will make them more suitable for plantation in high-density orchards. To carry out this work, a large availability of olive genes will be necessary. To date, about 400 sequences of olive have been deposited in GeneBank, that is, 109 nuclear sequences, 90 ribosomal, 136 cpDNA +mtDNA, 26 EST, 44 SRAP Markers and 16 retrotrasposoms (Rugini et al. 2006).

12.7.5 Cryopreservation of Olive Germplasm

As described in Section 12.5.1, numerous programmes are today ongoing aimed at the preservation of the large genetic variability of olive through the establishment of in-field collections. However, olive germplasm kept this way is costly and is vulnerable to losses due to diseases, pests, extreme environmental conditions and economic pressures. Hence, some research groups are at present involved in the exploitation of tissue culture technology as a possible alternative approach to the preservation of olive germplasm. Among the various methods available (see, e.g., Lambardi and De Carlo 2002), plant cryopreservation (i.e., the storage of explants at the ultra-low temperature of liquid nitrogen) seems to be the most promising for the long-term conservation of olive germplasm. Shoot tips of the cv Arbequina, for instance, showed 30% survival after recovering from the storage in liquid nitrogen, provided that they were previously desiccated to 30% of their original moisture content (Martinez et al. 1999). Lambardi et al. (2002) applied a procedure of 'vitrification and one-step freezing in liquid nitrogen' to shoot tips excised from in vitro-grown shoot cultures of the cv Frantoio. Following the recovery of explants from liquid nitrogen and their plating in a regrowth medium, 15% survival rate was achieved but only from shoot tips that had been obtained from apical buds. With a similar procedure, promising results have been recently obtained with the cryopreservation of shoot tips from the Italian cvs Gentile di Larino and Ascolana Tenesa (Nisi et al. 2006). Unlike the 'vitrification' technique, the application of the 'encapsulation-dehydration' procedure was not effective in the protection of either 'Frantoio' (Benelli et al. 2001b) or 'Arbequina' (Martinez et al. 1999) explants during ultra-rapid freezing.

Alternatively to shoot tips, embryogenic cultures of olive proved to be a suitable material for cryopreservation using the 'vitrification' approach (Lambardi

et al. 2002). The technique was applied to portions of embryogenic masses of the cv Canino, containing somatic embryo primordia at different stages of development. After their recovery from the storage at −196°C, almost 40% of the cryopreserved embryogenic samples survived and promptly recovered to proliferate. Moreover, the recovered embryogenic cultures showed enhanced proliferative and morphogenetic activity, and the embryo primordia that were present in the embryogenic masses before cryopreservation greened when transferred into the light and developed rapidly to the cotyledonary stage. It must be emphasized that, due to the possibility of somaclonal variation occurrence during long-term culture of dedifferentiated cells, somatic embryos cannot be considered the best material for germplasm conservation. However, as evidenced in Section 12.7.4, the embryogenic cultures are a very important tool for genetic transformation studies and the possibility to store them in liquid nitrogen prevents the decline of embryogenic potential due to repeated subculturing.

12.8 Conclusions

The demand for olive oil is increasing in the world, not only for its gastronomic importance but also for its recognised value for human health, making it the 'king' of the typical Mediterranean diet. Hence a concomitant increase of the world production of high-quality olive oil is not only desirable and expected in the near future, but also to induce a positive effect in the price of extra-virgin olive oil, which is still often much higher than alternative vegetable fats, such as those from peanut, soybean, maize and sunflower. The economical aspect has particular importance for the Mediterranean countries where the olive is often among the most important cultivated fruit species. Hence the major olive-growing countries are presently deeply involved in an important work of transformation of the old groves (made of large and ancient trees) into modern, intensive and mechanisable orchards. It is obvious that a fundamental contribution is expected by the presently ongoing breeding programmes, as well as from the further development of strategies for the characterisation, the propagation and the conservation of genetic resources, all actions requiring the continuous support of international organisations and national institutions. This broad-spectrum activity is a requirement for the production of new cultivars which must drive the olive towards the 'new era' of intensive and mechanised orchards. The consequent improvement of yield and quality of olive productions in turn will make more remunerative the activity for the olive growers and, at the same time, will produce a beneficial effect on the market price of olive products.

An important contribution is also expected from the application of biotechnologies to olive, in terms of both the production of high-quality plants for new orchards and the creation of improved cultivars by genetic transformation having reduced size, superior rooting ability, resistance to abiotic and biotic stresses. In addition, the characterisation of cultivars by molecular markers (using RFLP, RAPD, AFLP and SSR techniques) as well as the safe conservation of genetic resources by non-conventional (cryopreservation) approach will also give their contribution to olive

improvement. To speed up this work, traditional breeding and biotechnological approach have to move in synergism, to drive the modernisation of olive culture in the 3rd millennium.

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Part III
Industrial Crops

Chapter 13
Breeding *Hevea* Rubber

P.M. Priyadarshan, P.S. Gonçalves, and K.O. Omokhafe

13.1 Introduction

Hevea brasiliensis (Willd. ex Adr. de Juss.) Muell.-Arg., the prime source of commercial rubber, is a deciduous perennial tree of the family Euphorbiaceae (Fig. 13.1). The predominant constituent of rubber derived from *Hevea* is cis-1,4 polyisoprene $(C_5H_8)_n$ where n may range from 150 to 20,00,000 (Pushparajah 2001). The invention of vulcanization by Goodyear in 1839 adjudged rubber as a prime raw material that was otherwise unknown to mankind for over 450 years, since Christopher Columbus gave the first description of rubber in the fifteenth century (Priyadarshan and Clément-Demange 2004). It staked almost 40% of the export revenue of Brazil till 1940 (Dean 1987). However, Brazil and adjoining countries of Latin America share only 2% of the production due to the infestation of South American Leaf Blight (SALB-*Microcyclus ulei* (P. Henn. von Arx.). The Southeast Asian countries enjoy dominance in rubber production and trade by contributing more than 90% of the 7.97 million tons of rubber produced worldwide in 2003 (Sekhar 2004). Thailand with 2.3 million tons is at the helm followed by Indonesia, India, Malaysia, China, Vietnam, Côte d'Ivoire, Liberia, Sri-Lanka, Brazil, Philippines, Cameroon, Nigeria, Cambodia, Guatemala, Myanmar, Ghana, D.R. of Congo, Gabon and Papua New Guinea.

13.2 Commercial Importance

Rubber is the strategic raw material for more than 40,000 products, including 400 medical devices (Mooibroek and Cornish 2000). Primarily due to its molecular structure and high molecular weight (> 1 million daltons) it has resilience, elasticity, abrasion resistance and impact resistance that cannot easily be obtained by artificial polymers. Search for alternative sources of natural rubber resulted in

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Fig. 13.1 A view of rubber plantation

experiments on industrial exploitation of guayule (*Parthenium argentatum* Gray) as a source of high quality latex. Although economic considerations may prevent commercial exploitation of new rubber-producing microorganisms, transgenic yeasts and bacteria may yield intermediate or alternative (poly-) isoprenes suitable for specific applications.

13.3 Botanical Aspects

Rubber is synthesized in over 7500 plant species, confined to 300 genera of seven families, namely, Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae (Cornish et al. 1993). The genus *Hevea* includes 10 species (Table 13.1) (Webster and Paardekooper 1989; Wycherley 1992). A few species are inter-crossable (Clément-Demange et al. 2000). Consequently, the *Hevea* species can be considered as a species complex. Since an elaborate description of taxonomical and botanical aspects of *Hevea* is out of scope of this article, readers may refer other sources (Schultes 1977a, 1987; Wycherley 1992; Priyadarshan 2003a; Priyadarshan and Gonçalves 2003; Priyadarshan and Clément-Demange 2004) for narrations on the subject. The natural habitats of *Hevea* species are Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Surinam and Venezuela (Webster and Paardekooper 1989). All species are diploids having $2n = 36$ chromosomes ($x = 9$), with the exception of one triploid clone of *H. guianensis* ($2n = 54$) and the existence of one genotype of *H. pauciflora* with 18 chromosomes (Baldwin 1947). However, *Hevea brasiliensis* behaves as amphidiploid (Ramaer 1935; Ong 1975; Wycherley 1976; Priyadarshan and Gonçalves 2003). All species were probably evolved in Amazonian forests over 100,000 years (Clément-Demange et al. 2000).

Table 13.1 Occurrence and features of *Hevea* species

Species	Notable features[1]
H. benthamiana Muell.-Arg.	Complete seasonal defoliation Medium size tree. Habitat: swamp forests
H. brasiliensis (Willd. ex. Adr. Juss.) Muell.-Arg.	Complete defoliation. Medium to large tree size. Habitat: well drained soils
H. camargoana Pires	Possibility of natural hybridization with *H. brasiliensis*. 2–25 m tree height Habitat: seasonally flooded swamps
H. camporum Ducke	Retains old leaves until new leaves appear. Maximum 2 m tall. Habitat: dry savannahs
H. guianensis Aublet	Retains old leaves until new leaves and inflorescences appear. Grows at higher altitudes (1100 m msl) Medium size tree Habitat: well drained soils
H. microphylla Ule	Complete defoliation. Small trees. They live on flooded area (igapós). Habitat: sandy soils
H. nitida *Mart. ex Muell.-Arg*.	Inflorescences appear when leaves are mature. Small to medium size trees (2 m).
H. pauciflora (Spr. ex Benth.) Muell.-Arg.	Retains old leaves until new leaves and inflorescences appear. No wintering. Small to big size trees. Habitat well drained soils, rocky hill sides.
H. rigidifolia (Spr. ex Benth.) Muell.-Arg.	Retains old leaves even after inflorescences appear. Small tree from savannahs. Sometime tall, with small crown on the top. Habitat: well drained soils
H. spruceana (Benth.) Muell.-Arg.	Retain old leaves until new leaves and inflorescences appear. Flowers reddish purple. Medium size tree Habitat: muddy soils of islands
H. paludosa Ule[2]	*Small leaflets, narrow and thin in the fertile branches; Habitat: marshy areas*

After Wycherley (1992), Schultes (1977a), Gonçalves et al. (1990), Pires (1973), Pires et al. (2002) Brazil (1971).

[1] Deciduous characteristics mentioned here have a bearing on the incidence of fungal diseases especially through secondary leaf fall (*Oidium*) since retention of older leaves may make the tree 'escape *oidium*'. Dwarf types are desirable for the possible wind tolerance. All species are diploid (2n = 36) (Majumder 1964), and are inter-crossable (Clément-Demange et al. 2000).

[2] Pires (1973), Pires et al. (2002) considered 11 species including *H. paludosa*; Brazil (1971) considers 11 species.

(Modified from Priyadarshan and Gonçalves 2003).

13.4 Historical Aspects

Fusée Aublet was the first to give a botanical description of the genus *Hevea* in 1775. Five distinguished men played pivotal role for rubber domestication, namely,Clement Markham (of British India Office), Joseph Hooker (Director of Kew Botanic Gardens), Henry Wickham (Naturalist), Henry Ridley (Director of Singapore Botanic Gardens) and R. M. Cross (Kew Gardner). Kew Botanic Gardens played the crucial role for rubber procurements and distribution. As per directions of Markham, Wickham collected 70,000 seeds from Rio Tapajoz region of Upper Amazon (Boim district) and transported them to Kew Botanic Gardens during June 1876 (Wycherley 1968; Schultes 1977b; Baulkwill 1989). Of the 2,700 seeds germinated, 1,911 were sent to Botanical Gardens, Ceylon during 1876, where 90% of them survived. Later, during September 1877, 100 *Hevea* plants specified as 'Cross material' were sent to Ceylon. However, in June 1877, '22 seedlings', not specified either as Wickham or Cross, were sent from Kew to Singapore, which were distributed in Malaya and formed the prime source of 1,000 tappable trees found by Ridley during 1888. An admixture of Cross' and Wickham's materials might have occurred, as the '22 seedlings' were unspecified (Baulkwill 1989). Seedlings from Wickham's collection of Ceylon were also distributed worldwide. But somehow, rubber trees covering millions of hectares in Southeast Asia are believed to be derived from very few plants of Wickham's original stock from the banks of the Tapajoz (Imle 1978). After reviewing the history of rubber tree domestication into East Asia, Thomas (2001) drew the conclusion that the modern clones have invariably originated from the 1,911 seedlings sent to Ceylon in 1876. Hence, the contention that the modern clones were derived from '22 seedlings' is debatable. Moreover, if the modern clones are derived from 1,911 seedlings, then the argument that they originated from a 'narrow genetic base', as believed even now, needs to be reviewed.

P. J. S. Cramer conducted experiments on variations observed among 33 seedlings introduced from Malaysia in 1883 from which the first clones of East Indies were derived (Dijkman 1951). Along with van Helten, a horticulturist, he could standardize vegetative propagation by 1915. The first commercial planting with bud-grafted plants was undertaken during 1918 in Sumatra's east coast. Ct3, Ct9 and Ct38 were the first clones identified by Cramer (Dijkman 1951; Tan et al. 1996). Commercial ventures gradually spread to China, Thailand, India, Sri Lanka and Vietnam and rubber became an integral part of the economy of Southeast Asia toward latter half of the 20th century. Around 1950, bud grafted clones proved to be overwhelmingly popular because of higher productivity.

Progress in yield improvement in *Hevea* resulted in a gradual increment, from 650 kg/ha in unselected seedlings during 1920s to 1,600 kg/ha in best clones during 1950s. The yielding potential was further enhanced to 2,500 kg/ha in PB, RRIM (Malaysian), RRII (Indian), RRIC (Sri Lankan), IRCA (Côte d'Ivoire), BPM (Thai), NIG (Nigeria), IAC (Brazilian) and RRIV (Vietnamese) clones during 1990s. During these 70 years of rigorous breeding and selection, notable clones like RRIM 501, RRIM 600, RRIM 712, PB 217, PB 235, PB 260, RRII 105, RRIC 100, IRCA

18, IRCA 230, IRCA 331, BPM 24, IAC 35 and IAC 40 were derived (Tan 1987; Simmonds 1989; Clément-Demange et al. 2000; Priyadarshan 2003a; Priyadarshan et al. 2005; Omokhafe and Nasiru 2005). Primary clones selected during the aforesaid period (PB 56, Tjir 1, Pil B84, Pil D65, Gl 1, PB 6/9 and PB 86) became parents of improved clones. It must also be acknowledged that primary clones like GT 1 and PR 107 are still widely used although their identification traces back to the 1920s.

13.5 Propagation Systems

Rubber is currently planted in the form of grafted trees, at a density of about 450 trees per hectare. It experiences an immature phase varying from 5 to 9 years, depending on climate, soil conditions and management. Propagation through grafting enables the multiplication of elite genotypes as clones. The high level of homogeneity in bud-grafted trees should exhibit intra-clonal variation in yield to a minimum, barring factors such as (a) soil heterogeneity, (b) difference in juvenility of buds and (c) variable seedling rootstocks. On the contrary, such clonal populations exhibit significant variations. In an experiment with RRII 105, total volume of latex and dry rubber yield ranged between 5.0 to 325.0 ml and 1.8 to 144.0 g, respectively (Chandrashekar et al. 1997). The differences exhibited are significant and refutable for a homogeneous population. Due to the lack of an efficient cloning technique, the root system directly affects soil–plant relationships, such as water and mineral uptake, water stress resistance and resistance to wind uprooting (Ahmad 2001). Moreover, efficient breeding for growth of budded clones and the increasing use of fast growing clones may have generated an imbalance between stock and scion, so emphasizing the uprooting hazard (Clément-Demange et al. 1995). Consequently, cloning the root system is a major challenge for rubber tree breeding, as it would greatly facilitate growth, yield improvement and adaptation to various environments. Rubber trees can be propagated as seedlings also, for the polyclonal seedlings are desired plant materials for non-traditional areas (Sasikumar et al. 2001).

13.6 Laticifer System

Hevea has articulated laticifers issued from the anastomose of latex cells in newly formed parts of the tree, forming a paracirculatory ramified structure (syncytium). This laticifer system is notably developed in the soft bark of the trunk from which latex can be extracted by tapping (Figs. 13.2 and 13.3). The laticifers successively generated by the cambium are organized in cylindrical rings that are not interconnected. There are no plasmodesmata between the latex vessels, or between them and their surroundings (de Faÿ and Jacob 1989); there are also no associated companion cells in contrast with sieve tubes. Connections exist between the laticifer systems of the stock and the scion, evidenced by the transport of latex (Bonner

Fig. 13.2 Tapping Hevea rubber in Amazonian forests

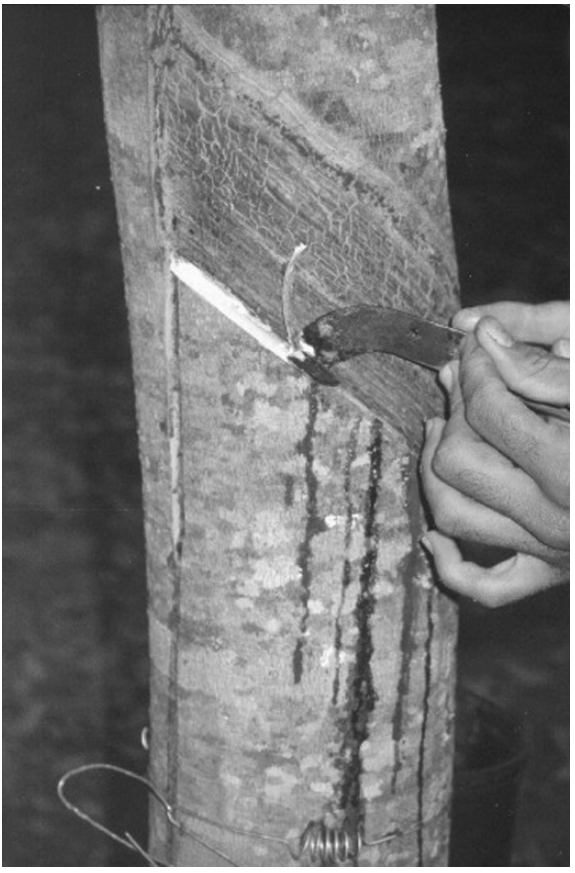


Fig. 13.3 Refined commercial tapping of bud grafted tree

and Galston 1947). The latex is a cytoplasm that contains predominantly rubber particles, as well as lysosomal microvacuoles known as lutoids; it also contains double-membrane organelles rich in carotenoids, which look similar to plastids but their role has not yet been fully elucidated (Paardekooper 1989). On tapping, nuclei and mitochondria remain adhered to the plasmalemma, and consequently, cannot be found in the latex, which makes possible latex regeneration after tapping. Rubber particles, made of clustered polymer chains, are surrounded by a phospholipoglycoprotein monomembrane with outside negative charge that ensures the colloidal stability of the latex before coagulation and protects the rubber chains against oxidative degradation.

Coagulation at the level of the tapping cut is an important limiting factor of rubber yield as it stops latex flow. Lutoids with coagulating factors, such as hydrolytic enzymes, other proteins and many ions with positive charge, cause the rupture of their membrane and play a key role in this process. High osmotic gradient near the cut also is an adding factor that leads to the plugging of laticifers (Southorn and Edwin 1968). Plugging index, a ratio between initial and final flow of latex, shows differences between clones (Milford et al. 1969) and can be used as a selection variable. Turgor pressure in the laticifers, as high as 10–14 atmospheres before sunrise, is vital for the flow of latex. Water plays a key role in latex flow after tapping and all the ecophysiological factors that affect water balance and water flux in the tree influence latex flow and coagulation.

Regeneration of the latex and rubber between two tappings is related to the cellular metabolism of the laticifer system and with the ecophysiological functioning of the tree. The full regeneration of the latex after one tapping was estimated to be around 72 h (Serres et al. 1994). Assimilation (photosynthesis), transport of sugars, and allocation to the different competing sinks play a key role in the regeneration of the latex. One of the most striking features of tapping is that it generates a direct competition for carbohydrate assimilates between latex regeneration and the whole growth of the tree. This can easily be observed by the strong reduction in growth increments in trunk growth and in tree height, a few weeks after the beginning of tapping. Faster growth resumes when tapping is stopped. This partition of assimilates has been theoretically modelled by Simmonds (1982), based on some ideas of Templeton (1969). As such, assimilate partitioning has become vital especially when rubber wood is getting increasing importance (Fig. 13.4).

The intensity and the duration of latex flow and latex regeneration determine latex yield. Ethephon stimulation delays coagulation and prolongs latex flow in laticifers. This is a clonal response and is a genetic component of yield potential, which can be subjected to selection and breeding. A review (d'Auzac et al. 1997) took stock of the physiological functioning of the laticifer system and its response to stimulation, and presented the concept of the physiological typology of the clones, which is based on the metabolic activity of the laticiferous cells, the provision of these cells with sucrose and their protection from oxidative stress. Based on this concept,a biochemical diagnosis (the "latex diagnosis") was developed from the measurements of the dry rubber content, sucrose ratio, inorganic phosphorus ratio and thiol ratio in the latex (Jacob et al. 1995; d'Auzac et al. 1997). Gohet et al. (1996) fine-tuned this

Fig. 13.4 Rubber wood ready for chemical impregnation

methodology. Further, it was envisaged that latex diagnosis could be used to detect physiological stresses (Lacote et al. 2004).

The current best clones, with annual yield little more than 2,500 kg of latex per hectare appear still far below the yield summit (4,000 kg) estimated by Templeton (1969). This has provided encouragement to rubber breeders to continue their efforts in genetic improvement.

13.7 Breeding Objectives

Improving dry rubber yield is the exclusive objective of *Hevea* breeding. Growth of the trunk during immature phase, yield per tree over a specific period, stability of the stand per unit area and resistance to stresses (tapping panel dryness, wind damage, varied diseases, low temperature, higher altitude and moisture deficit) are some of the factors that govern productivity levels. Latex yield and growth are not correlated, obviously due to differential partitioning of assimilates. Breeding and selection are exclusively applied to scion and the choice of rootstock is very limited. The possibility of cloning the whole plant *in vitro* would allow breeding to be applied to the root system for resistance to root diseases, for better adaptation to specific soils and for anchorage. This leads to the concept of 'compound tree' with three different genetic components, namely roots, trunk and canopy, each selected for its own requirements (Simmonds 1985, 1989). High yielding trunks with canopies resistant to SALB have been experimented by the way of crown budding, but it failed commercially.

Adaptation and yielding potential of clones to specific environments are optimized through multi-location trials and localized experimentation. Characterization of the architecture of the trees in connection with wind risk and phenology is assessed in relation to susceptibility to leaf diseases (*Colletotrichum gloeosporioides* Penz. Sacc., *Microcyclus ulei*) are vital (Priyadarshan et al. 2001). Studies on adaptation of clones to new environments, especially to sub-optimal or marginal

areas, are gaining momentum (Priyadarshan 2003a,b). In all these aspects, diversification of clones allows large plantations to mitigate risks. Among those clones, the more stable ones are identified for recommendation to small holders, since small holders represent a predominant share. A selection focused on fast growing trees with effective competence toward weed growth, canopy adapted to multi-cropping, clones adapted to uneven and intensive tapping systems and climatic variations needs to be exercised.

Derivation of clones for timber has emerged as a recent objective. An estimation from RRIM shows that a hectare of rubber plantation can yield around 190 m^3 of rubber wood, and 2.7 million m^3 of *Hevea* wood would be available from Malaysia (Arshad et al. 1995). Also, there is some interest generated among the scientists to evolve rubber as a factor producing useful chemicals, especially life saving drugs (Yeang et al. 2002). Possibilities of using rubber trees for reforestation or carbon sequestration may come up in future, which breeders may have to take up with required priority.

13.8 Genetic Resources and Variability

Hevea brasiliensis is believed to be an amphidiploid ($2n = 4x = 36$) that got stabilized during the course of evolution. This contention is amply supported by the observance of tetravalents during meiosis (Ong 1975). However, for practical purposes, *Hevea* is considered as a diploid genus ($2n = 2x = 36$). In situ hybridization studies revealed two distinct 18S–25S rDNA loci and one 5S rDNA locus, suggesting a possible allotetraploid origin with the loss of 5S rDNA during the course of evolution (Leitch et al. 1998). Hence, as long as a potential ancestor with $2n = 18$ is not known, rubber tree would be considered as an amphidiploid (Priyadarshan and Gonçalves 2003). Locus duplications are infrequent in *Hevea* genome, and they could have occurred due to chromosomal modifications posterior to the polyploidization event (Seguin et al. 2003). Consequently, the two ancestral genomes of *Hevea* would have strongly diverged. Only a comprehensive molecular analysis along this objective will reveal the details of origin.

Allied species of *Hevea* make up a gene pool for breeding purposes, especially for the identification and introduction of genes of resistance to leaf diseases (Priyadarshan and Gonçalves 2003). Within *Hevea brasiliensis*, a clear distinction needs to be made between 'Wickham' population and the 'Amazonian' population. While the Wickham population was domesticated and bred for more than a century, Amazonian populations are still under evaluation and despite poor yield, they display a fairly high resistance to leaf diseases, such as *Microcyclus* or *Corynespora cassiicola* Berk et. Curt. Wei. (Clément-Demange et al. 2000).

During 1951–1952, 1,614 seedlings of five *Hevea* species (*H. brasiliensis, H. guianensis, H. benthamiana, H. spruceana* and *H. pauciflora*) were introduced in Malaysia (Tan 1987). Brookson (1956) has given an account of these introductions. In Sri Lanka, 11 clones of *H. brasiliensis* and *H. benthamiana*, and 105 hybrid materials were imported during 1957–1959, through triangular collaboration of USDA, Instituto Agronomico do Norte (IAN), (Brazil), and Liberia. Many of these clones

were later given to Malaysia (Tan 1987). During 1981, due to initiative taken by the International Rubber Research and Development Board (IRRDB), 63,768 seeds, 1,413 m of bud wood from 194 high yielding trees, and 1,160 seedlings were collected from Brazilian Amazonia (Gonçalves 1981; Tan 1987; Simmonds 1989). This collection was carried out over three states, namely, Acre, Rondonia, and Mato Grosso, from 60 different locations spread to 16 districts. Of this, 37.5% of the seeds were sent to Malaysia and 12.5% to Côte d'Ivoire. Half of the collections were maintained in Brazil. The accessions from budwood collection were brought to Malaysia and Côte d'Ivoire after quarantine against SALB. After the establishment of two IRRDB Germplasm Centers in Malaysia and in Côte d'Ivoire, other IRRDB member countries were supplied with material according to their request.

Attempts to improve the yield of wild accessions through Wickham × Amazonian crosses resulted in recombinants with low yield, ranging between 30 and 50% of the level of GT1, probably due to the important genetic gap lying between the two populations. Conversely, a wide variability was found within these crosses for growth, with probable heterotic effects enabling the selection of very vigorous Wickham × Amazonian clones. It is quite evident that the Wickham population, though originally meager in number, was subjected to natural pre-breeding. This must have occurred in two ways, one through indirect selection of ortets exhibiting adaptation to specific hydrothermal environment and the other by evaluation of useful recombinants. A clear difference in branching habit could be observed between accessions from Acre and Rondonia, which more often have tall trunks with poor branching located at great heights and those from Mato Grosso that display trees with abundant branching at low heights (Clément-Demange et al. 1998). Furthermore, during 1995 an expedition was launched by RRIM to collect rubber seeds from Brazil. From this collection, about 50,231 seedlings were planted in Malaysia, including allied species (RRIM Annual Report 1997; MRB Annual report 1999).

13.9 Breeding Methodologies and Achievements

Breeding methodologies utilized for maximizing genetic gain are based on breeding objectives with the specific aim of providing farmers with high yielding clones. Such methodologies are backed by the theory of quantitative genetics, which derives clones well adapted to a given environment. Elements of breeding methodologies are available with major contributions of Dijkman (1951), Shepherd (1969), Wycherley (1969), Tan (1987), Simmonds (1989), Clément-Demange et al. (2000); Priyadarshan (2003a) and Priyadarshan and Clément-Demange (2004). These ideas are discussed here with a separate section on biotechnology.

13.9.1 Primary Clones

The first clones released out of seedlings were those of Cramer's *Cultuurtuin* (Ct3, Ct9, Ct88) selected from 33 seedlings planted in Penang through Java in Indonesia

(Dijkman 1951). Mixed planting of these clones gave yield over 1,700 kg/ha, against unselected seedlings (496 kg/ha) (Tan et al. 1996). During 1924, Major Gough selected 618 seedlings from a population of about 1 million in Kajang district of Malaysia that yielded prominent primary clones like Pil A44, Pil B84, Pil B16, PB 23, PB 25, PB 86, PB 186 and Gl 1 (Tan et al. 1996). By 1930, it was understood that the primary clones had reached a plateau of yield (Tan 1987). Hence, the emphasis shifted from primary clones to recombinants issued from controlled pollination (Fig. 13.5). Simultaneously, polyclonal seed gardens were organized with improved clones for raising polyclonal seedlings for ensuring supplementary planting materials. Thus, the best seedlings came from Prang Besar Isolated Gardens (PBIG), Gough Gardens (GG), and Prang Besar Further Proof Trials (Tan et al. 1996). By 1970, polyclonal seedling areas extended to 7,700 hectares. Both yield and secondary attributes were given deserving importance while selecting clones based on 65% and 35% scores for yield and secondary attributes respectively (Ho et al. 1979; Tan et al. 1996). The procedure involved field selection in the estates, nursery selection applied to seedlings, small-scale selection with 16 plants per genotype, and large scale testing with 128 plants per genotype.

Polyclonal seed gardens involving clones with high general combining ability (GCA) ensures panmictic conditions for deriving seedlings with high genetic divergence. Selection for both vigor and high yield can be exercised in such seedlings (Simmonds 1986). After popularization of clones in 1950s, the potentiality of extending rubber to marginal areas was realized. This seems to be an appreciable option since results on the yield of polyclonal seedlings from non-traditional areas like Tripura (northeast India) and Konkan (western India) are encouraging (Sasikumar et al. 2001; Chandrashekar et al. 2002). There is a contention that yield and girth variation can be largely accounted for by additive genetic variance (Gilbert et al. 1973; Nga and Subramaniam 1974; Tan 1981). As per general genetic principles, selection based on genotypic values as reflected by GCA would be more

Fig. 13.5 A view of recombinants (hybrids) of Hevea rubber

reliable and desirable. GCA could be estimated by the evaluation of seedling progenies, in order to select the best parent clones. DNA fingerprinting can contribute significantly to assess molecular diversity of parents and their progenies. Optimum number of parents is crucial while constituting seed gardens and Simmonds (1986) suggested that a lay out involving nine clones with all hetero neighbors as the best.

The extent of selfing due to lack of self-incompatibility may reduce the vigor of first generation population (SYN_1), since there is no evidence of self-incompatibility. Since inbreeding reduces zygotic ability to germinate, the presumption is that only cross-pollinated seeds will survive (Simmonds 1986). Till recently, such SYN_1 progenies were considered as Class I planting material in Malaysia and must be of better use in non-traditional/marginal areas. However, factors like agronomic performance of such synthetic seedlings, the long time taken to attain seed production and supply of seeds in tune with demand need to be evaluated before utilizing this methodology. In contrast, seed gardens can be viewed as a recombination tool for addressing the improvement of wild Amazonian populations, where the ability to flower and set seed make the major criteria to ensure maximum genetic combinations (Fig. 13.6). With this view, 50 Amazonian parents were analysed in Côte d'Ivoire using microsatellite markers (Blanc et al. 2001). Most of the paternal contribution to the progenies was due to a restricted number of male parents with substantial flowering, hence were very far from a panmictic status. It is implicit that each seed garden need to be evaluated with DNA fingerprinting.

13.9.2 Derivation and Evaluation of Recombinants

Recombination breeding starts with production of full-sib families, followed by Seedling Evaluation Trial (SET), Small Scale Clonal Trial (SSCT) and Large Scale Clonal Trial (LSCT) with selection practiced at every level. This process is cyclical, with the best clones becoming candidates for recombination in the next cycle. Yield improvement from 500 kg/ha in primary clones to 2,500 kg/ha in the current clones could be attained through recombination breeding and selection in RRIM and Prang Besar (Malaysia). RRIC 100 series released in Sri Lanka during 1970s is yet another example. Much of the hybridisation work in Malaysia, Indonesia, India, Côte d'Ivoire, Brazil, Thailand and Vietnam further strengthened the array of hybrid clones with differential genetic set-up, obviously due to selection pressure applied under varied conditions (Table 13.2).

At least 16 primary clones are considered prime progenitors of many modern clones (Fig. 13.7). However, many valuable recombinants must have been lost during the course of assortative mating of primary and hybrid clones followed by subsequent directional selection for yield under varied geo-climates (Priyadarshan 2003a). The crossing of 'the best with the best' (GAM, Generation-wise Assortative Mating), with strong emphasis on selection for precocious yield within Wickham material (Wycherley 1976) has been practiced in all these recombination breeding

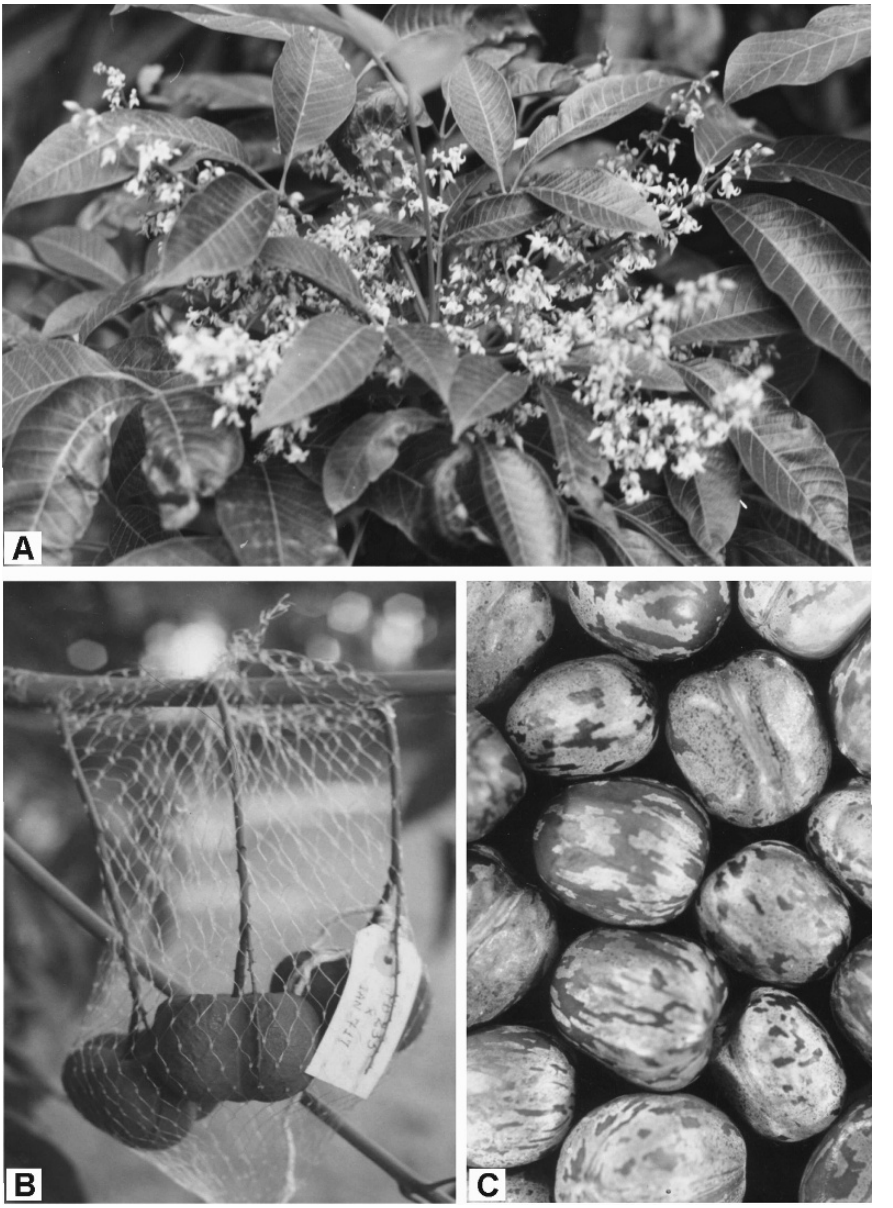


Fig. 13.6 (**A**) Flowers; (**B**) Bagged fruits borne from hand pollinated flowers; and (**C**) seeds

programs. Breeding for disease resistance has to take account of specific aspects related with host × pathogen interactions. But this exercise has to go a long way before it achieves clones combining resistance and higher yield. Interestingly, in the clones developed in Nigeria, the cross involving one primary and a secondary clone ended with high yielders, which are under evaluation (Table 13.3).

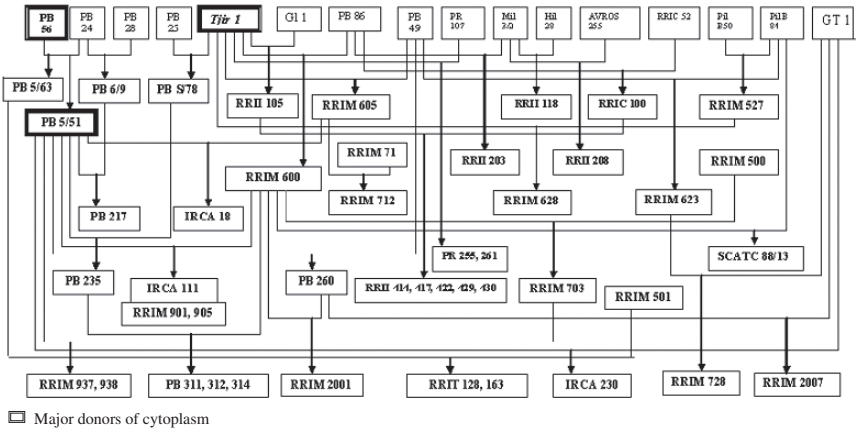
Table 13.2 Profile of prominent clones

Clone	Parentage	Yield (kg/ha)	Girth increment during tapping	Resistance to						
				Wind damage	Panel dryness	Pink Disease	Oidium	Colletotrichum	Corynespora	Phytophthora
RRII 105[I]	Tjir 1 × Gl 1	2,210	3	3	5	5	3	5	5	1
RRII 203[I]	PB 86 × Mil 3/2	1,618	4	3	2	3	3	NA	3	3
RRII 208[I]	Mil 3/2 × AVROS 255	1,587	3	3	3	NA	3	NA	NA	NA
RRIC 100[M]	RRIC 52 × PB 83	1,774	3	5	3	3	4	3	5	NA
RRIM 600[M]	Tjir 1 × PB 86	2,199	4	4	4	1	3	3	1	1
RRIM 623[M]	PB 49 × PB 84	1,622	4	2–3	3	2–3	1–2	3–4	4	1
RRIM 712[M]	RRIM 605 × RRIM 71	2,264	2	5	4	3	3	1	3	3
RRIM 936[M]	GT 1 × PR 107	2,146	3	4	3	4	3	4	4	2
RRIM 937[M]	PB 5/51 × RRIM 703	2,483	2	5	3	4	3	3	5	3
RRIM 2015[M]	PB 5/51 × IAN 873	2,760	4	NA	NA	NA	4	4	4	3
PB 217[M]	PB 5/51 × PB 6/9	1,778	4	4	4	2	2	3	4	1
PB 235[M]	PB 5/51 × PB S/78	2,485	3	2	2	3	2	2	4	3
PB 255[M]	PB 5/51 × PB 32/36	2,283	3	4	2	2	2	2	4	2
PB 28/59[M]	Primary clone	2,023	1	3	3	2	2	2	4	2
PR 255[M]	Tjir 1 × PR 107	2,018	3	4	3–4	3	1	3	4	3
PR 261[M]	Tjir 1 × PR 107	1,838	3	4	3–4	3	1–2	4	3	3
IRCA 111[CD]	PB 5/51 × RRIM 600	1,446	5	3	3	NA	NA	NA	NA	NA
IRCA 230[CD]	PB 5/51 × GT 1	1,807	5	3	3	NA	NA	NA	NA	NA
RRIT 163[I]	PB 5/51 × RRIM 501	2,086	2	NA	NA	NA	3	NA	3	NA
HAIKEN 1[C]	Primary clone	1,500	3	4	3	2	NA	NA	NA	NA

Table 13.2 (continued)

Clone	Parentage	Yield (kg/ha)	Girth increment during tapping	Resistance to						
				Wind damage	Panel dryness	Pink Disease	Oidium	Colletotrichum	Corynespora	Phytophthora
REYAN 8–333[C]	SCATC 88–13 × SCATC 217	2,187	3	3	3	NA	3	NA	NA	NA
BPM 24[M]	GT 1 × AVROS 1734	1,394	2	3	3	3	3	2	3–4	4
IAN 873[B]	PB 86 × FA 1717	1,920	4–5	3	4	NA	4	4	NA	NA
IAC 301[B]	RRIM 501 × AVROS 1511	2,320	4	4	4	NA	4	4	NA	NA
IAC 300[B]	RRIM 605 × AVROS 353	887	3	2	2	NA	3	2	NA	2
Fx 3864[B]	PB 86 × PB 38	1,755	4	3	3	NA	2	2	NA	3
IAN 4493[B]	EX 441 × Tjir 1	1,711	3	3	2	NA	2	2	NA	2
IAC 303[B]	RRIM 505 × AVROS 1511	2, 190[6Y]	3	3	2	NA	2	2	NA	2
PB 260[VN]	PB 5/51 × PB 49	1, 691[10 Y]	4	3–4	3–4	3	4–5	4	NA	3–4
RRIC 121[VN]	PB 28/59 × IAN 873	1, 654[10 Y]	4–5	4	4	4	2	3–4	NA	4
GT 1[VN]	Primary clone	1, 459[10 Y]	3	5	5	3	2–3	3	NA	3–4
RRIV 4[VN]	RRIC 110 × PB 235	2, 103[10 Y]	2	2	4	3–4	2–3	2	NA	4

1 = poor; 2 = below average; 3 = average; 4 = good; 5 = very good. NA = Not available, since the disease is not prominent.
Under conditions of M = Malaysia; I = India; C = China; CD = Cote d'Ivoire; B = Brazil.
Tapping system = s/2 d/2 6d/7 86%; No. of tapping days per year = 158 ± 11 (with wide regional variation depending on weather); Trees per hectare = 327 ± 34.
IAN 873 exhibits good tolerance to SALB.
Tapping under Vietnamese (south east) conditions =S/2 d/3 6d/7.
6 Y=average over 6 years; 10 Y=average over ten years.
REYAN is new name for SCATC.



Gl Glenshiel, Malaysia; GT Gondang Tapen, Indonesia;
IRCA Institute de Recherches sur le Caoutchouc en Afrique, (Côte d'Ivoire);
Mil Milakande, Sri Lanka; RRIC Rubber Research Institute of Ceylon (currently Sri Lanka);
RRII Rubber Research Institute of India; RRIM Rubber Research Institute of Malaysia;
RRIT Rubber Research Institute of Thailand; Tjir Tjirandji, Indonesia; PB Prang Besar, Malaysia;

Fig. 13.7 Parentage of outstanding clones

13.9.3 Genetic Resource Management

Yield in rubber analyzed according to different types of mating designs was shown to have a large additive genetic variance. Heritability and general combining abilities for yield and growth have been investigated at the RRIM and are high, thus justifying GAM (Gilbert et al. 1973; Nga and Subramaniam 1974; Tan 1977, 1978, 1981; Simmonds 1989). Importance of low female fertility of many parents emerges here as a limiting factor for producing every full-sib progeny. The need for selecting highly heterozygous clones and reducing the risk of narrowing the genetic base are the two prime attributes that need attention (Simmonds 1989). One option is to involve Amazonian germplasm in breeding programs. Although such crosses appear as the best way to introgress the new germplasm into breeding populations, most of the Amazonian genotypes bear a large part of alleles unfavorable for yield (genetic burden in heterozygous plants). Pre-breeding appears necessary within the Amazonian groups before using them as progenitors in crossing with Wickham (Baudouin et al. 1997; Priyadarshan and Clément-Demange 2004). Since a detailed evaluation of whole *Hevea* germplasm is quite impossible, a working population of 287 accessions was extracted at the IRRDB African Germplasm Centre (Clément-Demange et al. 1998). It was proposed to combine the use of field experiments and molecular markers (microsatellites) for extracting a clonal population of reduced size (core collection) containing maximised genetic variability (Hamon et al. 1998; Brown 1989; Clément-Demange et al. 2000).

Table 13.3 Twenty four clones of *Hevea brasiliensis* developed in Rubber Research Institute of Nigeria (RRIN)

	Class 1 clones*				Class 2 clones**			
	National Code	RRIN Code	Parentage Mean Yield		National Code	RRIN Code	Percentage	Mean Yield
1.	NIG 800	RRIN C 76	RRIM 501 × Har 1	2679	NIG 901	RRIN C 289	PB 5/51 × PR 107	3528
2.	NIG 801	RRIN C 83	RRIM 600 × PR 107	2229	NIG 902	RRIN C 292	PB 5/51 × PR 107	3351
3.	NIG 802	RRIN C 114	RRIM 501 × RRIM 628	2014	NIG 903	RRIN C 291	PB 5/51 × PR 107	3237
4.	NIG 803	RRIN C 48	RRIM 600 × PR 107	2765	NIG 904	RRIN C 367	PB 5/51 × RIM 600	3233
5.	NIG 804	RRIN C 1	RRIM 600 × Tjir 1	3207	NIG 905	RRIN C 227	RRIM 501 × RRIM 628	3152
6.	NIG 805	RRIN C 15	RRIM 628 × RRIM 501	1944	NIG 906	RRIN C 380	PB 5/51 × RRIM 600	3069
7.	NIG 806	RRIN C 163	RRIM 501 × RRIM 628	2723	NIG 907	RRIN C 321	RRIM 501 × Har 1	3043
8.	NIG 807	RRIN C 145	RRIM 501 × RRIM 628	2699	NIG 908	RRIN C 366	PB 5/51 × RRIM 600	3033
9.	NIG 808	RRIN C 143	RRIM 501 × RRIM 628	2411	NIG 909	RRIN C 369	PB 5/51 × RRIM 600	3009
10.	NIG 809	RRIN C 150	RRIM 501 × 628	2388	NIG 910	RRIN C 368	PB 5/51 × RRIM 600	3000
11.	NIG 810	RRIN C 159	RRIM 501 × RRIM 628	2383				
12.	NIG 811	RRIN C 154	RRIM 501 × RRIM 628	2334				
13.	NIG 812	RRIN C 162	RRIM 501 × RRIM 628	2312				
14	NIG 813	RRIN C 202	RRIM 600 × PR 107	2090				

Source: Omokhafe, and Nasiru, I. (2005) Genetic improvement of *Hevea brasiliensis* in Nigeria. International Natural Rubber Conference, Cochin, India, pp. 13–17.

*: Tested in multilocation trials.

**: Undergoing multilocation trials.

13.9.4 Selections

Breeding cycle in rubber extends to 20–30 years between pollination and yield assessment, distributed over three selection stages. This justifies standardization of early selection methods to optimise and shorten the cycle as much as possible. One component of early selection is identification of traits at young age that have correlated response with yield at maturity and the other is combined management of different selection stages to improve the accuracy of estimation of genetic value. Several parameters, namely, girth, height, bark thickness, latex vessel number, latex vessel and sieve tube diameters, and rubber hydrocarbon in bark and petiole were inconsistent in having relations with yield both at seedling and mature stages (Gunnery 1935). Also, parameters like quantity of latex oozing out of leaflets or petiolules, plugging index, photosynthetic rate, number of stomata (Senanayake and Samaranayake 1970; Ho 1976; Zhou et al. 1982; Samsuddin et al. 1987) were studied but only plugging index and latex vessel number showed consistent and significant correlations with yield (Huang et al. 1981). Hénon and Nicolas (1989) showed that thickness of the bark cannot be considered as a reliable attribute to predict yield but the number of latex vessel rings can help to differentiate poor yielders in both Amazonian and Wickham populations. Recently, Sreelatha et al. (2004) demonstrated ATP concentration in latex as a possible indicator of high yield. However, this relationship needs to be confirmed in high yielding clones under varied environments, due to differential yielding pattern of clones under different environments. The first stage of selection is applied to full-sib seedlings (SET, Seedling Evaluation Trial) and information from this stage is used for selecting new clones to be evaluated as grafted trees in Small Scale Clone Trial (SSCT) (Fernando and de Silva 1971). Analysis of different procedures for assessing yield on young seedlings confirmed the use of only mild selection at the first nursery stage (Gnagne et al. 1998; Tan 1978).

For combining SET and SSCT, Gnagne et al. (1998) studied the relationships between the two stages of early selection. A combined family × individual selection was proposed in the form of a linear combination of family value and individual values. At nursery stage, with only one seedling tree per genotype, it will almost be impossible to directly assess the environment effect, so limiting the predictive efficiency of this first stage. With this view, early selection does not aim for priority to shorten the cycle but to improve the selection efficiency. Alternately, molecular genetic markers are considered independent of the environment and using them as predictors can contribute to improve the accuracy of genetic value assessment according to the concept of marker-assisted selection or MAS (Lynch and Walsh 1998). But this technique needs further refinement. The third stage of selection is Large Scale Clonal Trial (LSCT), involving evaluation of individual genotypes in sets and on rather large plots over a long period at different locations. Further, Priyadarshan and Clément-Demange (2004) proposed an alternate method, where the seedlings raised at a moderate spacing will be evaluated at maturity and then cut back for bud wood multiplication. The high yielders will be evaluated directly

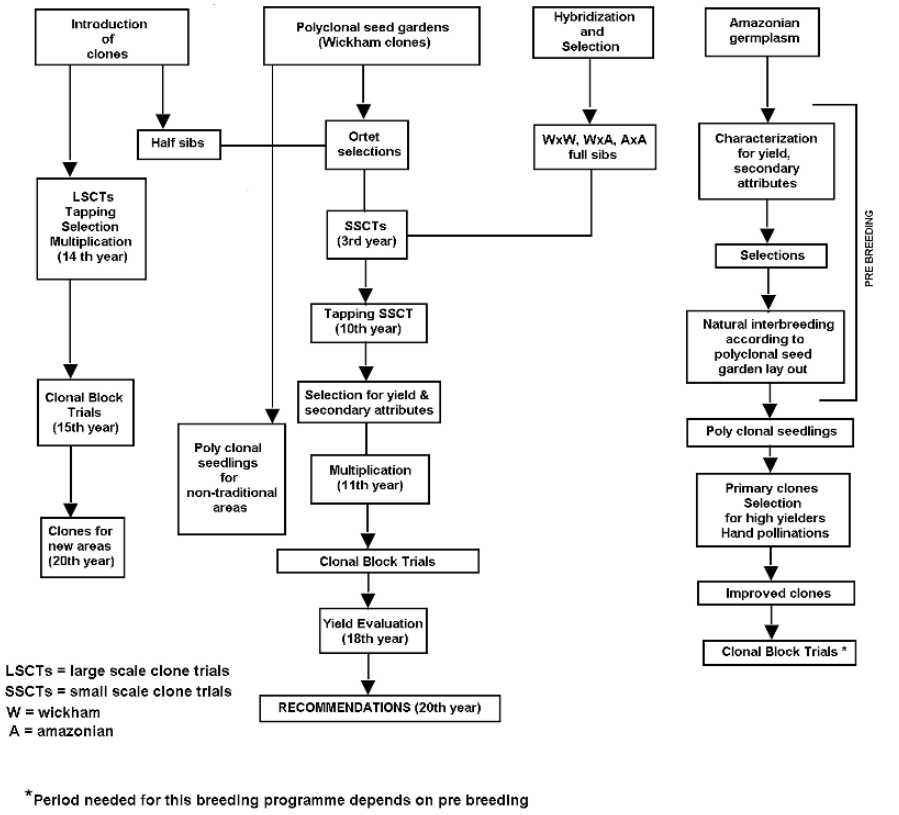

Introduction of clones
Polyclonal seed gardens (Wickham clones)
Hybridization and Selection
Amazonian germplasm
Half sibs
Ortet selections
WxW, WxA, AxA full sibs
Characterization for yield, secondary attributes
LSCTs Tapping Selection Multiplication (14 th year)
SSCTs (3rd year)
Selections
PRE BREEDING
Tapping SSCT (10th year)
Natural interbreeding according to polyclonal seed garden lay out
Clonal Block Trials (15th year)
Selection for yield & secondary attributes
Poly clonal seedlings
Poly clonal seedlings for non-traditional areas
Multiplication (11th year)
Primary clones Selection for high yielders Hand pollinations
Clones for new areas (20th year)
Clonal Block Trials
Improved clones
Yield Evaluation (18th year)
Clonal Block Trials *
LSCTs = large scale clone trials
SSCTs = small scale clone trials
W = wickham
A = amazonian
RECOMMENDATIONS (20th year)
*Period needed for this breeding programme depends on pre breeding

Fig. 13.8 Scheme for breeding clones

as LSCT before they are recommended for a specific location. This can reduce the experimental period from 34 to 20 years (Fig. 13.8).

13.9.5 Breeding Against Stresses

The increased global demand for rubber prompted the countries outside the hitherto traditional zone to focus their attention on the cultivation of rubber (Pushparajah 1983). Rubber was also extended to sub-optimal environments of the countries coming under the traditional belt. This is mainly due to three reasons, namely, increasing demand for rubber, crop diversification under traditional areas and efforts to upgrade the living standards of the people under the so-called sub-optimal environments (Priyadarshan 2003a). Specific areas of China, Thailand, Vietnam, India, Côte d'Ivoire and southern plateau of Brazil fall under sub-optimal environment (non-traditional areas) that experiences one or more stress situations, namely, drought,

low temperature, high altitude, diseases and strong winds. Latitudinal range will be more than 10 °N or S of the Equator (Table 13.4). On the other hand, the traditional rubber growing tracts extend up to 10 °N and S of equator, and offer environmental conditions ideal for rubber cropping. They are: (a) 2,000–4,000 mm rainfall distributed over 100–150 days per annum (Watson 1989); (b) mean annual temperature around 28 ± 2 °C with a diurnal variation of about 7 °C (Barry and Chorley 1976) and (c) sunshine hours of about 2,000 h per year at the rate of 6 h per day in all months (Ong et al. 1998). In a study with hydrothermal index, Rao et al. (1993) rationalized Senai of Malaysia (1° 36′N; 103° 39′E) to be the most suitable area for rubber cultivation and production.

Latitudinal increase will imply fall in mean annual temperature and more prominent winter conditions during November – January. North-eastern states of India, the highlands and coastal areas of Vietnam and south China that lie between 18° and 24 °N are regions well recognized as inhospitable for the crop, exhibiting stress situations like low temperatures and typhoons (Zongdao and Yanqing 1992; Priyadarshan and Gonçalves 2003). It may also be worthwhile to note that rubber areas of China and Tripura fall under the same latitude range, though climatic conditions in vivid pockets of China shall vary because its tropical and sub-tropical regions are undulating and diversified (Priyadarshan 2003a). Southern plateau of Brazil, especially São Paulo (20–22 °S; 450–500 m MSL) is a prominent rubber area. This move to grow rubber seasonally affected by dry and cold conditions is mostly motivated to escape from the climatic conditions congenial to SALB. These areas, apart from high altitude, offer high rainfall that often exceeds the basic requirements. North Côte d'Ivoire is also being experimented with rubber, where warm climatic conditions prevail (Dea et al. 1997). A geo-climatic comparison of various environments with Tripura, China, Brazil, Côte d'Ivoire, Indonesia, Vietnam and Thailand would amply reveal a spectrum of climatic conditions over which rubber is grown. In India, marginal areas delineated as non-traditional zones, spread over to the states of Maharashtra, Orissa, Tripura, Assam, West Bengal, Meghalaya and Mizoram, pose a multitude of hazards, namely, moisture stress, low temperature, wind, high altitude and disease epidemics, apart from altered soil physical properties (Priyadarshan 2003a). Adaptation of existing clones to non-traditional environments with clone specific/area specific tapping schedules and fertilizer inputs are of prime importance to achieve latex yields compared to favorable zones.

13.9.6 Stress Factors and Yield

Low temperature, wind, diseases, climatic changes due to higher altitude and latitude are the stress factors influencing rubber culture. In China, two types of cold damages (chilling injury) have been identified, namely, radiative and advective (Zongdao and Xuequin 1983). In radiative type, the night temperature falls sharply to 5 °C, whereas the day temperature ranges between 15 and 20 °C or above; in advective type, the daily mean temperature remains below 8–10 °C, with a daily

Table 13.4 Geo-climatic feature non-traditional rubber areas of India, Vietnam, China and Brazil

Attributes	India (Agartala)	Vietnam (Pleiku-Highlands)	Vietnam (Dong Hai-Coastal)	China (Hainan Island)	China (Xishuangbanna, Yunnan)	Brazil (Pindorama-Sao Paulo)
Temperature						
Annual mean	30.5	21.8	24.6	23–25	20.9–21.7	22.68
Coldest month mean	17.5	13.3	16.0	16.2–28.3	15.2–15.7	19.5
Extreme minimum	3.8	5.7	7.7	1.4–5.1	1.3–3.7	−2.1
Annual precipitation	1818.0	2272	2159	960–2400	1200–1535	1390
No. of days with rain	129	154	135	95–200	165–193	116
Penman ET_o (mm/day)	3.39	3.1	3.3	–	–	–
Relative Humidity (%)	80–85	80	83	79–86	83–86	64.1–80.3
Wind speeds (m/s)	1.38	2.8	2.8	1.2–4.5	0.5–0.8	1.5
Maximum wind speed (m/s)	35	28	40	80	24	10
Sunshine (h)	2500–2600	2377	1750	1747–2662	1787.8–2152.9	2376
Latitude	22°56′ and 27°32′N	13°59′N	17°28′N	18°10′ and 20°10′N	21°08′ and 22°34′N	21°13 S
Longitude	91°10′ and 92°21′E	108°E	106°37′E	108°35′ and 111°03′E	99°57′ and 101°51′N	48°56′E
Altitude (m)	30	778	7	5.5–328.5	100–1180	562
Soil type	Laterite/Sandy loamy	Ferrasols on basalt	Ferrasols on schists	Latosol/latosolic red soils	Latosol/Latosoic red soils	Red yellow podzolic/ medium texture
Geomorphology	Hillock/low lying areas	Relatively flat	Hills/Flat coastal areas	Hills/Flat coastal areas	Hills/valleys	Hillocks/high altitude ranges

minimum of 5 °C. In both types, under extreme circumstances, complete death of the plant is the ultimate outcome. An analogous atmosphere prevails in northeastern states of India also. Reports from China point out that while clones GT 1 and Haiken 1 can withstand temperatures as low as 0 °C for a short span, SCATC 93–114 can endure temperature as low as −1 °C. The cold wave conditions in Tripura state (north-east India) can be conveniently classified as relating to the radiative type. Chinese clones like Haiken 1, SCATC 88-13 and SCATC 93-114 are being evaluated in Tripura. The yielding pattern shows Haiken 1 to be a high yielder among Chinese clones, as compared with RRIM 600, which is used as a local check. Though SCATC 93-114 is known for its cold endurance, it never shows considerable yield potential under the conditions of Tripura, at least during the initial stages on B0-1 panel (Priyadarshan et al. 1998a,b). China has also developed Zhanshi 86, a clone borne out of a random cross between SCATC 93-114 and Wuxing I_3 is cold enduring than SCATC 93-114 (Senyuan 1990). Further, clones like Zhanshi 306-15 (RRIM 600× Guangxi 6–68) give around 10 kg of dry rubber per tree. But these conditions will be tested at the block level. IAN 873, a SALB resistant high yielding clone developed in Brazil shows resistance to cold weather in China (Senyuan 1990) (Tables 13.5 and 13.6).

In India, areas between 15–20 °N of western and eastern side have been identified as non-traditional zones. For instance, the Konkan region of western India experiences long dry periods, high temperatures, low atmospheric humidity and zero rainfall between September and May with daytime temperatures ranging at 38–41 °C during summer months (with a maximum of 47 °C). Though it gets rainfall of 2,430 mm, the distribution is uneven (Devakumar et al. 1998). The atmosphere during summer results in high vapor pressure deficit. Almost an analogous situation prevails in the eastern part of India.

Wind is yet another abiotic stress influencing establishment and growth of rubber. Contributing to the drying effect of drought conditions, it induces regimes of long-lasting steady winds during the dry season in highlands of Vietnam. Wind speeds of 2.0–2.9 m/sec retard rubber growth and latex flow, and 3.0 m/sec or above severely inhibit normal growth. Wind over Beaufort force 10 (more than 24.5 m/sec) play havoc with branch breaks, trunk snaps and uprooting of trees, mainly prevalent in China, during June to October (Watson 1989). Studies in China revealed that clones PR 107 and Haiken 1 can be wind enduring, and PB 5/51 is wind enduring in Tripura (Priyadarshan et al. 1998a). Establishment of shelterbelts, consisting of fast growing and wind resistant species, is one remedial measure being followed in China (Zongdao and Xuequin 1983). But this exercise needs proof, taking into account their effects on total stand as well as the economy of their implementation and land occupation. Alternatively, adoption of judicial pruning of branches and induction of branches at lower height can reduce wind damage from 25.3 to 13.7% (Zongdao and Xuequin 1983). In Côte d'Ivoire, rubber plantations often experience wind damage due to storms occurring at the onset of the rainy season (April–May) (Clément-Demange et al. 1995). In coastal areas and high lands of Vietnam and Sao Paulo state of Brazil also, clones perform differently owing to greater GE interactions (Tables 13.7 and 13.8).

Table 13.5 Yield and secondary attributes of 20 clones being evaluated in Tripura

Clone	Stand (initial)	Girth (mature)	Yield (projected) kg/ha	Crop efficiency*	Wind damage	TPD	Oidium Incidence
RRII 5	Average	Low[1]	1,618 #	0.85	Moderate	Low	S
RRII 105	Good	Moderate[1]	1,635 #	1.0	Moderate	Low	S
RRII 118	Good	High[1]	1,484 #	1.07	High	Mild	MT
RRII 203	Good	Moderate[1]	2,021 #	1.14	Low	Low	T
RRII 208	Good	Moderate[2]	1,534 @	0.93	High	Very mild	MT
RRIM 600	Good	Moderate[1]	1,817 #	0.99	Low	Moderate	T
RRIM 605	Good	Moderate[1]	1,341 #	0.74	Moderate	Moderate	MT
RRIM 703	Average	Moderate[1]	1,741 #	1.21	Moderate	Low	T
RRIC 52	Average	Moderate[1]	1,013 #	0.51	High	Low	T
RRIC 105	Average	High[1]	1,164 #	0.59	High	Low	MT
PB 5/51	Good	Low[1]	963 #	0.74	Low	Mild	HS[$]
PB 86	Good	Low[1]	1,136 #	0.77	Moderate	Low	T
PB 235	Good	High[1]	2,248 #	1.34	Moderate	Moderate	HS
GT 1	Good	Moderate[1]	1,374 #	0.85	Low	Mild	MT
Gl 1	Good	Low[1]	644 #	0.44	Mild	Low	HS
HARBEL 1	Average	Low	739 #	0.58	Low	Low	HS
PR 107	Good	Good[2]	669 @	0.29	Very low	Mild	HS[$]
SCATC 88/13	Good	Good[2]	1414 @	0.67	Low	Moderate	MT
SCATC 93/114	Good	Good[2]	848 @	0.24	Medium	Very mild	MT
HAIKEN 1	Good	Good[2]	1,276 @	0.68	Medium	Mild	MT

[1]Over 13 years; [2]over nine years; * g/cm of the tapping cut; [$] with secondary infection; # BO II panel; @ BO I panel; Projected yield = g/tree/tap x no of tappings x total stand (350). S = susceptible, MT = moderately tolerant, T = tolerant, HS – highly susceptible.

Table 13.6 Yield and secondary attributes of some clones in China

Clone	Site	Girth	Yield kg/ha	Years of tapping	Wind damage	Cold damage	oidium Incidence	TPD	Stand
GT1	Yunnan	Moderate	1,257.2	9	–	Low	Moderate	Moderate	Commercial
RRIM600	Yunnan	Moderate	1,190.3	10	Moderate	Moderate	Moderate	Moderate	Commercial
PR107	Yunnan	Moderate	1,007.9	10	Very low	Moderate	Severe	Low	Commercial
GT1	West Guangdong	Low	994	9	–	Low	Moderate	Moderate	Commercial
93–114	West Guangdong	Low	980.3	9	–	Very low	Moderate	Low	Commercial
YUNYAN 77-2	Yunnan	Moderate	1,874.5	9	–	Low	Severe	Mild	Advanced trial
REYAN 88-13*	Hainan	Moderate	1,700	8	Moderate	Moderate	Severe	Moderate	Advanced trial
REYAN 7-33-97	Hainan	Moderate	1,910	9	Low	Low	Moderate	Moderate	Advanced trial
REYAN 8-333	Hainan	Moderate	2,187	7	Moderate	Low	Moderate	Moderate	Advanced trial
DAFENG95	Hainan	Moderate	1,509.6	8	Low	Low	Moderate	Low	Advanced trial
WENCHANG11	Hainan	Low	1,953.5	10	Very low	Moderate	Low	Moderate	Advanced trial
HAIKEN 1	Hainan	Low	886.6	10	Very low	Moderate	Severe	High	Advanced trial

Tapping systems: the first three tapping years: $s/2 \cdot d/3$, and without Ethylene stimulation, about 75 tapping days per year after first three years of tapping: $s/2 \cdot d/2$, and without Ethylene stimulation, about 110 tapping days per year; *erstwhile SCATC

Table 13.7 Main characteristics of clones under marginal areas of Vietnam Kontum Province (Highlands – 550 m a.s.l. grey soil)

Clone	Girth at opening	Girth (mature)	Yield over ten years in kg/ha (g/tree/tap)	Oidium infestation	Phytophthora leaf fall	TPD
GT 1	Moderate	Moderate	1,191 (46.4)	Moderate	Moderate	Moderate
PB 235	High	Moderate	1,607 (59.8)	Severe	Low	Moderate
PB 255	Moderate	Moderate	1,174 (56.2)	Moderate	Moderate	High
PB 310	Moderate	Moderate	1,659 (52.8)	Low	Low	Moderate
PR 255	Low	Moderate	1,191 (49.8)	Moderate	–	Moderate
PR 261	Low	Moderate	1,197 (64.2)	Mmoderate	–	High
RRIC 110	Hhigh	Moderate	1,558 (66.3)	Low	Moderate	High
RRIM 600	Moderate	Moderate	1,177 (57.9)	Low	High	Moderate
VM 515	Moderate	Moderate	1,539 (63.4)	Moderate	Hhigh	High

TPD = tapping panel dryness

Daklak Province (Highlands- 700 m a.s.l, basaltic soil)

Clone	Girth at opening	Girth (mature)	Yield over seven years (kg/ha)	Oidium infestation	Phytophthora leaf fall	TPD
GT 1	Moderate	Moderate	1,005	Moderate	Moderate	Moderate
PB 235	Moderate	Moderate	998	Severe	Low	Moderate
PB 310	Moderate	Moderate	1,065	Low	Low	Moderate
PR 107	Low	Moderate	669	Severe	–	Moderate
RRIC 110	High	Moderate	1,422	Moderate	Moderate	Moderate
RRIM 600	Moderate	Moderate	1,153	Low	High	Moderate
RRIM 712	Moderate	Moderate	1,170	Moderate	Moderate	Moderate
VM 515	Moderate	Moderate	1,056	Moderate	High	High
RRIV 1	Moderate	Moderate	1,236	Low	Moderate	Low

Quang Tri Province (Coastal region- 50 m a.s.l., basaltic soil)

Clone	Girth at opening	Girth (mature)	Yield over five years (kg/ha)	Oidium infestation	Phytophthora leaf fall	TPD
gt 1	Moderate	Moderate	966	Low	–	–
pb 235	High	High	1,368	Low	–	–
pb 310	High	High	1,005	Low	–	–
rrim 600	Moderate	Moderate	1,355	Low	–	–
lh 82/92	High	Moderate	1,281	Low	–	–

RRIV 1, LH 82/92 = clone bred by RRIV (after Priyadarshan et al. 2005).

13.9.7 Diseases

Despite having all favourable climatic conditions, South American Leaf Blight (SALB) prevents Latin America from developing rubber plantations and it represents a permanent major threat to rubber in Asia and Africa (Chee 1976; Dean 1987; Davies 1997). Breeding work mainly based on backcross technique was undertaken to incorporate resistance in high yielding clones. However, the efforts were in vain due to unknown polygenic nature of the attributes, high variability of the pathogen and multiple interactions between strains and clones (Rivano1997a,b). Simmonds

Table 13.8 Yield and secondary attributes of 20 clones being evaluated in the plateau region of São Paulo State

Clone	Stand (initial)	Girth[1] (mature)	Yield[1] (projected) kg/ha[5]	Crop efficiency[2]	Wind damage	TPD	Oidium incidence
RRIM 600	Good	Moderate	2,100[3]	0.83	Low	Moderate	Low
PB 235	Good	High	1,834[3]	1.10	Moderate	Mild	Severe
GT 1	Good	High	1,679[3]	0.95	Moderate	Moderate	Moderate
PR 255	Good	Moderate	1,700[4]	0.93	Moderate	Moderate	Mild
PR 261	Average	Moderate	1,973[4]	1.21	Moderate	Low	Mild
IAN 873	Good	Moderate	1,890[3]	1.02	Moderate	Low	Moderate
Fx 3864	Good	High	1,755[3]	1.07	Moderate	Low	Moderate
PB 330	Good	Moderate	1,980[3]	0.99	Low	Mild	Moderate
PB 217	Average	Low	2,100[3]	0.68	Moderate	Mild	Mild
PR 107	Good	Moderate	1,870[3]	0.55	Low	Mild	Moderate
IAC 35	Good	Moderate	2,100[3]	0.82	Low	Moderate	Low
IAC 40	Good	High	2,400[3]	1.20	Moderate	Moderate	Low
IAC 56	Average	Moderate	1,900[3]	0.95	Moderate	Moderate	Low
IAC 300	Good	High	2,200[3]	0.82	Moderate	Moderate	Moderate
IAC 301	Good	Moderate	2,100[3]	0.65	Low	Moderate	Low
IAC 302	Good	Moderate	1,800[3]	0.80	Moderate	Low	Moderate
IAC 303	Good	Moderate	5,191[3]	0.82	Low	Low	Low
IAN 4493	Average	High	1,711[3]	0.93	Moderate	Moderate	Low
RO 45	Average	High	1,500[3]	0.70	Moderate	Low	Low
IAN 3156	Low Average	Moderate	2,500[3]	0.60	Moderate	Moderate	Moderate

[1] Over seven years.
[2] g/cm of the tapping cut.
[3] Tapping system 1/2S d/3 6d/7 (with ethephon stimulation 2.5%).
[4] Tapping system 1/2S d/2 6d/7.
[5] Prospected yield = g/tree/tap × number of tapping × total stand (400) (after Priyadarshan et al., 2005).

(1990, 1991) argues that the pathotype-specific resistance (vertical resistance-VR) has resulted in catastrophic failures. Horizontal resistance (HR) should be more effective and durable (Rivano et al. 1989; Simmonds 1990). Amazonian germplasm with resistant sources is yet to be improved for yield. With these views, efforts have been reoriented toward the analysis of partial resistance components (Junqueira et al. 1990). Recently, the genetic determinism of the resistance source of *H. benthamiana* (F 4542), widely used in many former backcross programmes has been characterized by a genetic map (Lespinasse et al. 2000b).

Resistance to other diseases was studied in some detail. Clones with an early refoliation like AVROS 2037, RRIC 100, RRIM 600, or PB 260, can develop their new leaves before the onset of the rainy season, and so are able to escape incidence of *Colletotrichum gloeosporioides*. In contrast, the widely planted clone GT 1 with late defoliation has been seriously affected in many areas of Malaysia, Indonesia (Kalimantan) and Central Africa. This consequence of early defoliation on the resistance of some leaf diseases of rubber was successfully used for the development and implementation of artificial early defoliation by Ethephon® aerial spray in Cameroon and Gabon for escaping from *Colletotrichum* (Sénéchal 1986). *Corynespora* Leaf Fall Disease (CLFD) has become a major threat for rubber cropping in South-East Asia and West Africa. An escape strategy related with early defoliating clones or by the way of artificial defoliation is not operative. It was demonstrated that the fungus is acting through the emission of a toxin (*cassiicoline*) in the leaves (Onesirosan et al. 1975). Studies conducted under controlled conditions have not put evidence of a significant interaction between clones and strains, but GT 1 is known to be tolerant and PB 260 and RRIC 100 to be highly susceptible (Breton et al. 2000). *Oidium heveae* seems to be favored by rather cold conditions prevalent toward the onset of refoliation (Rajalakshmy et al. 1997). In a comparative study with clones of various geographic origins, SCATC 93–114, Haiken 1 and RRIM 703 were adjudged as resistant to *Oidium* in the traditional areas of India (Rajalakshmy et al. 1997; Alice et al. 2000). While studying sensitivity relationships between clones, Alice et al. (2001) confirmed these results and marked SCATC 93–114, RRIM 703, Haiken 1, RRII 208, RRII 5 and PB 310 as stable sources of resistance over the years. Molecular markers for resistance to *Oidium* are to be developed to augment breeding programs with a cautious approach since the cost effectiveness of this technique is yet to be proved.

13.9.8 Breeding for SALB Resistance

Microcyclus was first identified by K.Ule on wild rubber trees in 1900. Endemic to the Amazonian forests where *Hevea* trees are dispersed in small groves, SALB proved to become epidemic in monospecific plantations, and still stands as an impediment to the extension of rubber plantations in Latin America. Significant efforts to develop rubber plantations in Brazil were initiated by the Ford Motor Company in 1928 with the creation and management of Fordlandia Estate, with

Wickham clones from Asia. However, the severity of SALB attacks led Ford Company to initiate rubber breeding programs since 1937. This breeding programme, initiated by H.Weir and then conducted by C.H.T. Townsend Jr., was first devoted to the collection of resistant sources of Amazonia (like upper Amazon, Acre, Peru and Bolivia, whereas the accessions from the Tapajos river banks proved to be highly susceptible). A crossing program between these sources and Wickham clones was carried out for combining resistance to SALB and higher yield (creation of clones FX). One of the most widely used resistant parents was F4542 from the *Hevea benthamiana* while accessions from other species, notably *Hevea pauciflora* and *Hevea spruceana*, were also assessed. Very low productivity and disease tolerance of the seedlings from Amazonian origin was observed, and the very high susceptibility of the Wickham genetic stock was confirmed. Such efforts were also carried out by Goodyear in Costa Rica (Turrialba station), and by the IAN (Instituto Agronomico do Norte) in Belem (Brazil) since 1940. Since the beginning of 1960s, the new concept of resistance consisting of two forms, vertical and horizontal, was elaborated by Van der Planck (1968). Robinson (1971) described the host-pathogen relationship as a system that breeding should aim to stabilize. A genetic model of the host-pathogen interaction in the case of vertical resistance was proposed (Flor 1955). Polygenic, horizontal resistance is assumed to be expressed in a continuous distribution of the resistance level among the genetic material (quantitative resistance). Horizontal resistance, if only partial, could reduce the capacity of new fungus races to emerge within the pathogenic population. But this general concept had to be confronted with the specificity of the *Hevea/Microcyclus* relationships. Considering rubber and *Microcyclus*, RRIM developed a research program in Trinidad, and Chee (1976) demonstrated that most of the vertical resistances to *Microcyclus* were expressed in the form of a classical hypersensitive reaction. To identify the different existing virulence factors expressed by the host–pathogen interactions (with any race bearing one or many virulence factors), successive studies demonstrated an increasing number of races and progressively evidenced the wide genetic and pathogenic variability of these races (Holliday 1970; Chee 1976; Darmono and Chee 1985; Hashim and Almeida 1987; Junqueira et al. 1988, 1990; Rivano et al. 1989; Rivano 1997a). Virulence was defined in rubber as the compatibility of one *Microcyclus* race with one clone, whereas aggressiveness was defined as the severity of the infection produced by a virulent race on a susceptible clone. With these facts and these new concepts, breeders had to begin their task anew, in a more complex framework.

Breeding the rubber tree for a sustainable, horizontal, polygenic, race-nonspecific resistance to *Microcyclus* appears as mandatory for counteracting the dynamics of new virulent strains. Even if total resistance was unattainable, good tolerance would be appreciable. This effort requires the gathering of a wide genetic variability for resistance, and the use of different identified components of resistance. Some of these components are (1) incubation period (from infection to the first symptoms), (2) infection latency period (from infection to the first sporulation of conidia), (3) duration of the leaf susceptibility period, (4) number of lesions per

leaf area unit, (5) average diameter of the lesions, (6) conidia sporulation intensity (Rivano et al. 1989), and (7) stromata emergence period. Earliness of wintering can contribute partly to avoid the disease development at the resumption of the rainy season. In order to identify horizontal resistance non-specific of any race, it is necessary to check the resistance or tolerance of the clones at field level in different sites (with different sets of races), and/or in the laboratory by controlled inoculation with different types of races, or with polyvirulent races. Controlled inoculation in the laboratory can be applied to leaf discs only for testing virulence in a 'clone-race' couple, for assessing other factors related with the aggressivity of the virulent races, the use of plants in pots is necessary.

These general ideas and concepts on the nature of the resistance have been developed for rubber by many authors (Simmonds 1982; Wastie 1986; Rivano et al. 1989; Simmonds 1990). From these studies, there is no evidence that there could be a strong intrinsic negative genetic relationship between rubber yield and resistance to the disease. Probably, genetic sources of resistance lie in some wild populations of the *Hevea* germplasm, which in the absence of any previous selection, bears the genetic burden of many alleles unfavorable to rubber yield. One possible way could be to improve these Amazonian populations for yield with no introgression from the Wickham population, in order to maintain a high level of resistance. Although requiring a lot of time, the improvement of resistant Amazonian populations for yield might help to select economically viable clones after only one cycle of crossing with Wickham clones or with the best yielding Wickham × Amazonian tolerant clones. Although a long process, this strategy can gain support from genetic determinism of the existing resistance sources with the possible help of molecular genetics.

Since 1992, Centre de Cooperation Internationale en Recherche Agronomique pour le Développement (CIRAD), Michelin and Brazilian universities (Sao Paulo, Viçosa, Santa Cruz), commenced concerted efforts *Microcyclus* challenge. The CMB project ('CIRAD-Michelin-Brazil') were conducted at the field sites of Michelin in Bahia (formerly a Firestone estate) and Mato Grosso and in French Guyana (Garcia et al. 2002a, b). The Mato Grosso estate, located in an 'escape' area (Wickham clones can be cultivated there), is used for conservation of the collections, hand pollination and preliminary testing. The diversity of virulences was proved to be much higher in the Bahia estate than in the French Guyana. Consequently, Bahia estate became the main field experimentation site. The germplasm collected by Firestone and conserved at Bahia estate, together with the Amazonian accessions of 1981 IRRDB collection, were re-evaluated for resistance. The accessions from states of Acre and Rondonia (western) were resistant than those from the Mato Grosso Eastern (western) (Le Guen et al. 2000). Although yielding low, Amazonian clones such as MDF180AS have been identified as expressing horizontal resistance. Eight clones were isolated to be resistant to SALB in the experiments conducted at Bahia (Garcia et al. 2004). The first major resistance gene ever identified and localized on a genetic map is of a minor disease, *Phyllachora huberi* (Le Guen et al. 2000).

13.9.9 Tapping Panel Dryness (TPD)

TPD is a physiological anomaly resulting in the cessation of latex flow and reduction of the tapping stands. Attention was first drawn at the beginning of the twentieth century on seedlings (Rutgers and Dammerman 1914), a phenomenon called 'Brown Bast disease' that progressed with browning of the inner part of the bark, its necrosis and malformation (de Faÿ 1981; de Faÿ and Jacob 1989, Eschbach et al. 1989). There is to be a range of dryness, starting from without any sign of browning to complete dryness (Fig. 13.9). Some researchers assume that there is a progressive evolution from tapping cut dryness to brown bast, whereas others think that they are two distinct diseases differing in their origin. It was found that tapping cut dryness was reversible, depending on tapping intensity and with seasonal variations, whereas brown bast is quite irreversible and leads to the loss of tapped trees (Jacob et al. 1994).

Reversible over-exploitation-induced tapping cut dryness is observed when the exploitation intensity exceeds the physiological capability of the tree to regenerate the latex. Here, the latex cell metabolism is severely disturbed (Bealing and Chua 1972). Before the occurrence of the first visible symptoms (partial drying of tapping cut), sucrose and dry matter are seen to decrease and inorganic phosphorus to increase in the latex (Pakianathan et al. 1982, Sivakumaran et al. 1984) with impaired rubber synthesis (Krishnakumar et al. 2001a). Membrane destabilization leading to bursting of the lutoids and consecutive in situ latex coagulation has been proposed to be associated with the occurrence of an uncompensated oxidative stress within the latex cells (Chrestin et al. 1984, Chrestin 1989). If the surrounding tissues do not seem to be much affected (absence of necrotic symptoms), they show some alteration of their biochemical composition (Yusof et al. 1995, Krishnakumar et al. 1999), with accumulation of proline (Wickremasinghe et al. 1987), decrease in cytokinin (Krishnakumar et al. 1997) and increased bark respiration (Krishnakumar et al. 2001b).

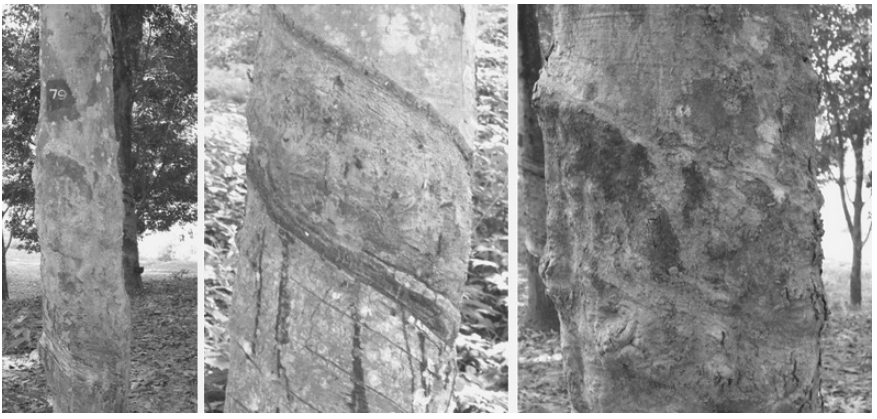


Fig. 13.9 Tapping panel dryness – various forms (*See* Color Insert)

Brown bast appears as the major visible problem as it contributes to the irreversible reduction of the tapped stand. Initial browning develops in the inner bark, seldom from stock-scion union (Jacob et al. 1994) and then necrosis appears on the outer part and spreads through the whole tapping panel. Since the syndrome spreads to neighboring trees, the possibility of a pathogen involvement has been investigated (Nandris et al. 1991a,b). A viroid has been recently claimed to be associated with this bark disease (Ramachandran et al. 2000). However, an alternative hypothesis that brown bast (as well as tapping cut dryness) is a physiological syndrome resulting from abiotic stress appears evident (Jacob et al. 1994; Faridah et al. 1996; Pellegrin et al. 2004). Though many hypotheses like localized soil characteristics in heterogeneous plots (Nandris et al. 2004), impaired cyanide metabolism, differences in the rates of cyanogenic compounds of leaves (Chrestin et al. 2004) have been put forward, none of these explain comprehensively the reason for panel dryness. In addition, terms like *tapping panel dryness, brown bast* and *bark necrosis* are to be classified and well defined wholly or separately to investigate the causes.

In the months just following opening, tapping cut dryness can be detected first but such cases can mask the development of real brown bast. In contrast, after two or three tapping years, regular census of trees affected by brown bast makes it possible to evaluate the development of disease. But clonal susceptibility to dryness seems to be related to the metabolic typology of clones. Clones with intensive metabolic activity and low sucrose reserves being less responsive to stimulation are more susceptible to dryness. Application of latex diagnosis to small scale clone trials (early stage of selection) could indicate dryness. The variation in clonal susceptibility is important and the ranking of clones seems to be roughly the same for the two syndromes, indicating thereby the possible occurrence of common causal factors. Studies on defense proteins suggested that a network of proteins (like chitinase and β-1,3-glucanase) are involved that may function as a biochemical barrier for invading harmful materials, thus becoming a governing factor for the onset of tapping panel dryness (Hao et al. 2004).

13.9.10 G × E Interactions

Yielding trends in Tripura (India), Vietnam and São Paulo (Brazil) that there are low and high yielding periods (Fig. 13.10). Under the hydrothermal situations of Tripura, in a study involving 15 clones of vivid geographical origin, almost all clones show an increment in yield toward the onset of cold season, that is, during October to November. Onset of cold season renders a stimulatory effect to maximize yield and the trend continues till the temperature falls below 15 °C during January. The clones are classified under two categories (a) one showing a slow escalation in yield from April onward, reaching the maximum during November, and receding sharply during December and January and (b) the other with a low yield regime during April to October, and with the peak yield during November and December (high yield regime), then receding during January. While PB 235 comes under the first category,

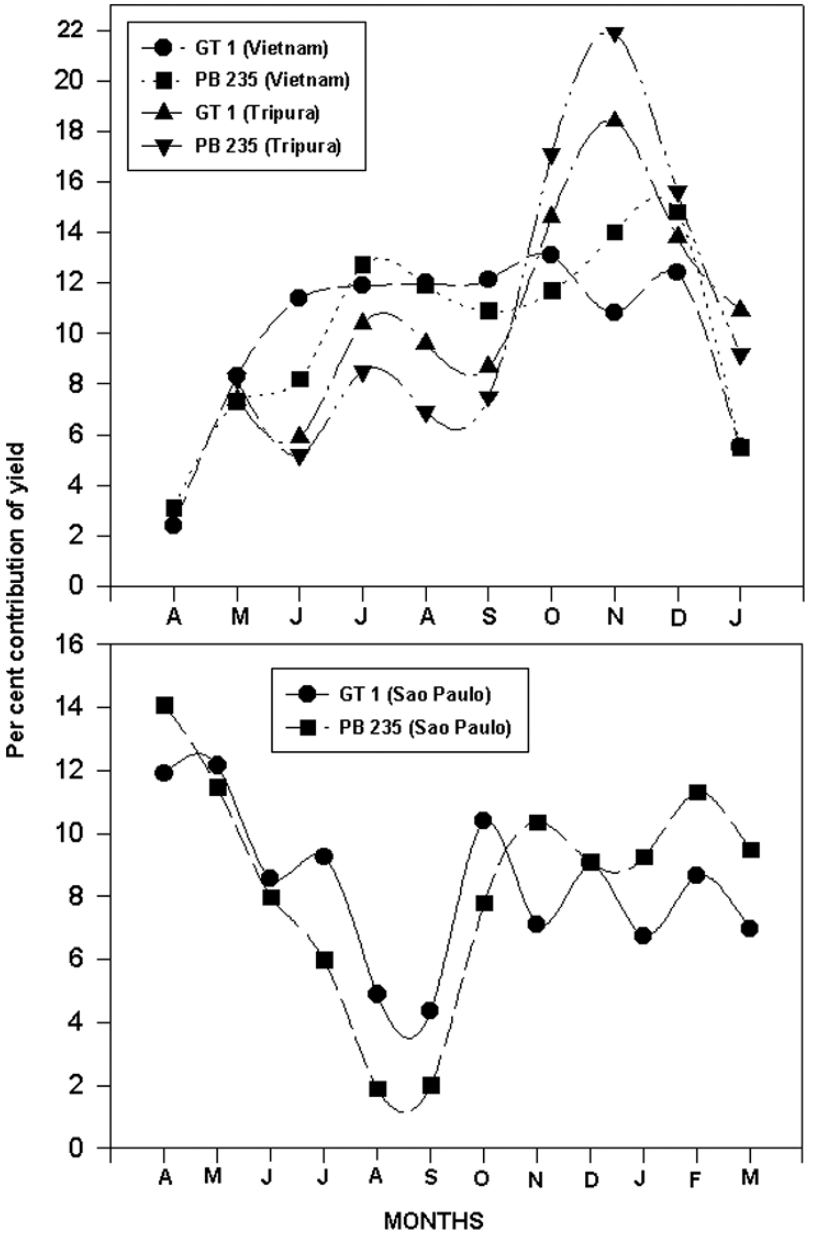

GT 1 (Vietnam)
PB 235 (Vietnam)
GT 1 (Tripura)
PB 235 (Tripura)
Per cent contribution of yield
A M J J A S O N D J
GT 1 (Sao Paulo)
PB 235 (Sao Paulo)
A M J J A S O N D J F M
MONTHS

Fig. 13.10 Contribution toward yield in GT1 and PB 235 over months in Vietnam (highlands), India (Tripura) and Brazil (Sao Paulo)

all the other clones come under the second. The trend shows that the first category is appreciable since the clones give considerable yield during Regime I, which ensures better returns to the planter. The rationale is that fall in temperature along with reduced evaporation and low wind speeds prevail upon the micro-environment to influence yield stimulation during October to December (Priyadarshan et al. 2000). The test of heterogeneity for environmental index showed high significance, so indicating that the high stability values of few clones (s^2i) over the years were due to linear effect of the climatic attributes (Priyadarshan 2003b). In São Paulo, RRIM 526 showed higher yield during low regime in comparison to RRIM 600 (Gonçalves, IAC, São Paulo, personal communication). These observations clearly rationalize the selection to be in favor of consistent yielder (Priyadarshan et al. 2000).

Under Malaysian conditions, Tan (1995) accounted GE interactions with a non-linear effect of wind damage and disease. In fact, these hazards play a prominent role in differentiating the adaptation of clones to one or different locations. Grouping of clones with high mean and low coefficient of variation is proved to be dependable in selecting better performers in a new environment (Tan 1995; Priyadarshan et al. 2002). GE interactions were also significant for rubber production and girth increment under the conditions of São Paulo (Gonçalves et al. 1998; Costa et al. 2000). In an investigation with seven clones (GT 1, PR 255, PR 261, IAN 873, RRIM 701, PB 235 and RRIM 600), over five environments, IAN 873 was adjudged as the most stable clone over years and locations (Gonçalves et al. 2003). Though GT 1 and IAN 873 were stable for girth and yield respectively, the change in rank of genotypes across the environments suggests that a breeding strategy of selecting specifically adapted clones in a mega environment will ensure the required productivity. Planters will also perceive yield stability as the most important socioeconomic aim to minimize crop failure, especially in sub-optimal environments.

13.10 Applications of In Vitro Culture

Long breeding cycle and larger size of the crop make the breeding process time consuming. Attainment of yield plateau prompted researchers to employ biotechnology tools to induce, increase and exploit new genetic variation. Biotechnology applied to *Hevea* can be discussed under two categories, namely, in vitro culture and molecular genetics. In vitro culture deals with regeneration and propagation, and molecular genetics involves identification, characterization, introduction and expression of novel genes.

Chua (1966) attempting derivation of callus from plumule tissues of seedlings was the first to attempt in vitro culture of rubber in 1960s. Further, the Rubber Research Institute of Malaysia took the initiative of maintaining callus cultures from various explants that later expanded to somatic embryogenesis and micropropagation through stem explants (Paranjothy and Gandhimathi 1976). While anther culture was employed to achieve pure lines followed by exploitation of heterosis, micropropagation and somatic embryogeny were worked out to have homogeneous

populations. Though research on in vitro culture commenced nearly 38 years back, even after rigorous experimentations, due to shortcomings toward commercial applicability, these areas are still under experimentation.

13.10.1 Anther Culture

Plants from *Hevea* pollen were initially made available during 1977 at the Baoting Institute of Tropical Crops, Hainan, China (Chen et al. 1979). Since then, at least four laboratories in China took the lead in researching production of haploid plants in vitro (Carron et al. 1989). Carron et al. (1989) enumerated three phases for the production of haploids from anther culture, namely, production of embryos, maturity of embryos and plant regeneration. Embryo production from callus takes nearly 50 days. The balance between callus development and initiation of embryos need to be maintained though use of MB medium with the judicious addition of NAA, coconut water, nitrogen, potassium and sugar for the production of calli and embryos (Chen 1984). The somatic callus then degenerates and the embryos develop from microspores. Sub-culture must be carried out at this stage into differentiation medium in order to ensure maturity of embryos (Chen et al. 1982). The cultures need 2–3 months for the apical bud to develop. Coconut water at this stage will be substituted with Gibberellic acid (GA_3) for better development of cotyledons. For plant regeneration, progressive increment of GA3, gradual withdrawal of other growth regulators, addition of 5-Bromouracil and reduction of sugar shall result in the development of plants from embryos. Cytological investigations of callus, embryos and plantlets showed mixoploidy (Qin et al. 1979). However, when the plants develop in vitro, there is a progressive tendency toward diploidy (Carron et al. 1989).

13.10.2 Somatic Embryogenesis and Meristem Culture

First plants from somatic embryogeny were obtained simultaneously in China and Malaysia from anther wall (Carron et al. 1989). Later, inner integument that represents mother tissue was used to produce somatic embryos by CIRAD in France (Carron and Enjalric 1982). The successive phases are callogenesis, differentiation, multiplication and plant regeneration. The judicious combination of 2,4-D, IAA and benzylaminopurine (BAP) and an increase in sucrose concentration promotes callogenesis under dark. Cultures are then taken to light with a changed macro-element composition to increase tissue proliferation (Carron et al. 1989). The differentiation medium is enriched with naphthoxyacetic acid (NOA) and BAP with low sucrose concentration. It takes 5–6 months in this medium for the embryos to develop. Carron et al. (1989) claim that nearly 3,000 globular or lanceolate embryoids could be achieved in 4 months. For plant regeneration, addition of indolebutyric acid (IBA) is crucial for promoting root and cotyledon formation. Successful plantlet formation

and acclimatization have been achieved in Haiken 1, Haiken 2 and SCATC 88/13 (Wang et al. 1980). Anther wall requires 2,4-D, NAA and kinetin (KN) for both callogenesis and embryo induction. BAP and zeatin are essential in addition to NAA and 2,4-D. GA_3 is found to increase the number of embryoids. BAP and GA_3 together with lower sucrose level are shown to improve plant regeneration (Sushamakumari et al. 2000). Carron et al. (1995a,b) gave a detailed account of the procedure and media formulation for somatic embryogenesis in *Hevea*.

Significant genotype-medium interactions are experienced in aforesaid procedures (El Hadrami et al. 1991; Montoro et al. 1993). Tissue-medium interactions are also very prominent. This is evident in integument culture, where a different additive of 234 mM sucrose, 9 mM BAP and 2,4-D were needed for embryogenesis. Abscisic acid (ABA) was essential for embryo development (Etienne et al. 1993; Veisseire et al. 1994a,b). Plant regeneration takes 25 days. Low germination percentage and plant conversion are seen as setbacks in this procedure, since the mechanism involved in this technology is poorly understood (Cailloux et al. 1996; Linossier et al. 1997). For instance, initiation and germination of embryos are seen to progress at higher temperature of 24–27 °C (Wang and Chen 1995; Wang et al. 1998). Polyethylene Glycol (PEG) and high $CaCl_2$ are seen to stimulate embryo production (Etienne et al. 1997a; Linossier et al. 1997). Thus, the clone-tissue-media interactions prevail in this technology that necessitates extensive basic studies. More recently, Etienne et al. (1997b) standardized a pulsed-air temporary immersion system for enhancing embryo production, through culturing embryogenic callus under immersion in an autoclavable filtration unit RITA™. Somatic embryo production was three to four times greater than those on a semi-solid medium, to the tune of 400 embryos per gram fresh weight with lesser number of abnormal embryos. Rubber Research Institute of Thailand (Bangkok) and CNRA (Côte d'Ivoire) planted 13,000 embryo-derived plants for field trials (Carron et al. 1995b). Clones PR 107 and PB 260 were highly regenerative. This is a leap toward regeneration of *Hevea* in vitro, since higher regeneration should be ensured to have homogeneous populations and rapid gene transfer system in *Hevea*.

Juvenile stem pieces are desirable for meristem culture that follows three phases, namely, primary culture, multiplication with rooting and acclimatization. Pretreatments with a mixture of Gentamycin, Kanamycin, Chlortetracycline, Chloramphenicol, Rifampicin and the fungicide Benomyl make the explants aseptic. Primary culture involves soaking explants in a solution of growth regulators (IBA and BAP) for 2–3 h. Budding is initiated in MB medium (Carron et al. 1989) without growth regulators. Isolated buds are cultured in half-strength of Lepoivre medium with IBA and BAP. These buds are sub-cultured to form micro-shoots that will in turn be cultured as explants in multiplication phase. Soaking base of the root in IBA-NAA mixture for 3–4 days induces roots. Rooted micro-cuttings can be transferred to soil in 4–5 weeks time. A number of clones like RRII 105, PB 5/51, PB 235, IRCA 438, IRCA 440, IRCA 442, PR 107 and GT 1 have been multiplied through micropropagation (Carron et al. 1995a). However, the acclimatization of plants is very crucial with a balance between relative humidity and temperature governing the establishment of plants in the soil.

Although gross experimentations were conducted for standardizing in vitro technologies, there had been many setbacks in commercializing these procedures (Carron et al. 1992). A number of aspects inherent in the explant tissue, namely, release of phenols, contamination of bacteria and fungi, recalcitrant status, reduced axillary branches, lack of sufficient juvenility, and above all, increased sensitivity of in vitro raised plantlets toward environmental attributes are responsible for the delay in commercialization. There are, however, remedial measures for these setbacks. Since the contamination of micro-organisms is location specific, newer chemicals are to be tried to raise aseptic cultures. Instead of treating the explants with anti-oxidants, the incorporation of the antioxidant in the media decreased browning (Seneviratne and Wijesekara 1996). The use of support systems like cellulose plugs in liquid media reduced synthesis of polyphenols, and embryogenesis activity could be maintained for more than 200 days (Housti et al. 1992). On the other hand, the growth regulators used to induce axillary branches and somatic embryogenesis are more or less the same throughout. Judicious combination of new growth regulators that have shown positive results in other tree species can be tried in rubber. Also, metabolism of ethylene and polyamines during callus development must be controlled by appropriate adjustment of growth regulators (Carron et al. 1992). More prominently, water status of embryogenic callus is a governing factor to enhance embryogenesis (Etienne et al. 1991). Further, Lardet et al. (1999) demonstrated that protein and starch accumulation commenced from 13th to 15th week, respectively, leading to development and maturity of zygotic embryos. However, the smaller size of somatic embryos that can accomplish relatively small mass of starch and protein reserves can lead to lower vigor and conversion rates where vigor is directly related to acclimatization success. Hence, increasing the size of somatic embryos through nutrient supplies deserve priority. To increase juvenility, air layering and progression to three to four generations can be exercised and explants from such source plants shall be used. If commercialized in the strict sense, these technologies can assist breeding programs and enhance productivity significantly.

13.11 Molecular Genetics and Breeding

Due to long generation time and larger size of the crop, new tools could be developed in order to manage germplasm variability and assist breeders in their recombination strategies. Molecular markers were developed that can be classified into three categories, namely, first generation (RFLPs, RAPDs and modifications), second generation mainly based on targeted PCR techniques with Simple Sequence Repeats (SSRs) or microsatellites, Amplified Fragment Length Polymorphism (AFLPs) and their modifications, and third generation markers like Expressed Sequence Tags (ESTs) and Single Nucleotide Polymorphism (SNPs) (Rudd et al. 2005). Though RFLPs are powerful for studying the genetic diversity and mapping, the technology is not preferred now since it is labor intensive, requires large DNA samples, and often involves radioisotopes. Its marker index value is also low (expressed as the

number of polymorphic products per sample) with only 0.10 compared to PCR-based marker systems like RAPDs (0.23), SSRs (0.60) and AFLPs (6.08) (Low et al. 1996). Ever since isozymes were utilized for clonal identification (Chevallier 1988; Yeang et al. 1998), tools like minisatellites (Besse et al. 1993a), RFLPs (Besse et al. 1993b, 1994), mtDNA RFLPs (Luo and Boutry 1995), RAPDs and DAFs (Low et al. 1996; Venkatachalam et al. 2001), AFLPs (Lespinasse et al. 2000a) and SSRs (Besse et al. 1993b; Atan et al. 1996; Low et al. 1996; Roy et al. 2004; Saha et al. 2005) were developed and used in detection and increment of molecular markers in *Hevea*. All marker systems, except SNPs have been applied in *Hevea* so far. The following section deals with various aspects of application of aforesaid techniques in dealing with measurement of molecular diversity, formulation of gene linkage maps, detection of QTLs, and evaluation of laticifer specific gene expression.

13.11.1 Molecular Diversity

Initial studies on isozymic diversity showed the existence of three genetic groups and many new alleles could be found in the Amazonian populations (Chevallier 1988), which was later confirmed through molecular studies (Seguin et al. 1996b). These studies indicate that the genetic diversity available in Amazonian accessions is immense that are yet to be utilized at the molecular level to enrich the Wickham population. However, transfer of such diversity can only be accomplished through gene manipulations at molecular level. Further, analysis of isozymes that are proteic genetic markers were developed at CIRAD through formulation of a diagnostic kit with 13 polymorphic isozymic systems for clonal identification along with a clonal identification database. This kit is proved to be able to differentiate a large set of cultivated clones (Leconte et al. 1994). However, the analyses are to be carried out near the field sites due to fragility of isozymes to varied temperatures, or otherwise, the samples need to be freeze-dried and transported to the laboratory. Hence, initiating molecular studies, Low and Bonner (1985) characterized *Hevea* nuclear genome as containing 48% of most slowly annealing DNA (putative single copy) and 32% middle repetitive sequences with remaining highly repetitive or palindromic DNA. The whole nuclear genome size was first estimated as 6×10^8 base pairs. Estimation with flux cytometry demonstrated 2×10^9 base pairs for *H. brasiliensis, H. benthamiana, H. guianensis, H. pauciflora*, and *H. spruceana* (Seguin et al. 2003).

Fingerprinting through RFLP minisatellite probes could be more powerful and identification of 73 Wickham clones was done with 13 probes associated with restriction enzyme *Eco*RI (Besse et al. 1993b). RFLPs were also used for identification of progeny with two common parents such as PR 255 and PR 261; RRIM 901 and RRIM 905; RRIM 937 and RRIM 938 (Low et al. 1996). Furthermore, Besse et al. (1994) using 92 Amazonian and 73 Wickham clones did an assessment of RFLP profiles. RFLPs, as molecular genetic markers, were used with homologous

probes from a CIRAD *Hevea* bank that showed genetic enrichment brought by Amazonian collections to *Hevea* germplasm, following genetic structure based on geographical collection sites (Besse et al. 1993a; Seguin et al. 1996b). Exceptionally, a clone from Rondonia (RO/C/8/9) showed eight specific restriction fragments and a unique malate dehydrogenase (MDH) allele, indicating its interspecific origin.

In a comparative analysis of SSRs of 20 clones of *Hevea brasiliensis* and six allied species of *Hevea*, Low et al. (1996) measured polymorphism in *H. pauciflora, H. guianensis, H. camargoana, H. benthamiana* and *H. brasiliensis*. Three microsatellite sequences of the gene for hydroxyl methyl-glutaryl-coenzyme A reductase 1 (HMGR-1) were polymorphic. Amplification of $(GA)_9$ region with appropriate primer converted these regions into sequence tagged microsatellite sites (STMS). Polymorphisms in STMS were with regard to band number and band length. While intraspecific polymorphism (in clones of *H. brasiliensis*) was mainly with number of bands, both number and length of bands contributed to interspecific polymorphism. The intraspecific polymorphism must be due to allelic differences arising from recombination. The interspecific polymorphism is the result of DNA insertion/deletion and point mutations. On the other hand, DAF profiles were very distinct for vivid species (Low et al. 1996). These polymorphisms must have played a role in delineating species during the course of evolution. Microsatellites that are tandem repeats of short (2–6 bp) DNA sequences are high utility markers that are codominant, highly polymorphic, abundant and uniformly dispersed in plant genome. It allows precise discrimination of even closely related individuals (Mallet 1995). In a bid to select suitable parents for extending rubber breeding programs, Lekawipat et al. (2003) applied microsatellites in detecting diversity in 40 Wickham and 68 Amazonian accessions. This was accomplished with 170 alleles from 12 microsatellite markers spread among all genotypes. On average, 14 alleles were available per locus. Wickham clones were unambiguously less variable than Amazonian accessions. Also, microsatellites of wild accessions are more polymorphic than cultivated Wickham clones and could be divided into three clusters depending on geographical origin of collections such as Acre, Rondonia and Mato Grosso. This conforms to the earlier studies on isozymes and RFLPs. Two clones (RO/OP/4 20/16 and RO/A/7 25/133) were unique as they do not fall under any cluster due to high level of specific alleles (Lekawipat et al. 2003). A microsatellite-enriched library was constructed in *H. brasiliensis* involving four types of simple sequence repeats like $(GACA)_n$ (10%), $(GATA)_n$ (9%), $(GA)_n$ (34%) and $(GC)_n$ (9%) (Atan et al. 1996). In cooperation with the French 'National Centre for Sequencing', CIRAD developed different microsatellite-enriched libraries in order to identify a large collection of microsatellite markers. Two possible applications are: clonal identification with the advantage of leaf samples sent through normal mail from one site to a distant laboratory and parental identification of seeds collected from an open pollinated seed garden (Blanc et al. 2001).

The evolution of cytoplasmic genome in *Hevea brasiliensis* was slower, due to lack of genetic recombination through meiosis. The estimated mean molecular size of chloroplast DNA (ctDNA) is 152 kb (Fong et al. 1994). Mitochondrial DNA

(mtDNA) was also analyzed with heterologous probes from broad bean by CIRAD and CNRA (Luo et al. 1995, Luo and Boutry 1995). A high mtDNA polymorphism was found in Amazonian accessions. The diversity of mtDNA of Wickham population is almost nil as only GT 1, a male-sterile clone, exhibited a different type from that of 49 other Wickham clones analysed. Mitochondrial DNA appears to be a valuable tool for studies on classification and phylogeny in plants, resulted more from DNA rearrangements rather than nucleotide substitutions (Palmer and Herbon 1988). Sequencing of a highly polymorphic mtDNA fragment from 23 genotypes showed real potential for phylogenetic analysis in *Hevea* (Luo and Boutry 1995). In chloroplast DNA analysis, much less polymorphism was found, therefore indicating the high level of conservation of this genome.

As a synthesis of these diversity studies, the *Hevea* genetic structure clearly appears as geographically structured (Besse et al. 1994), in relationship with the hydrographic network of Amazonian forest (Luo et al. 1995 and Seguin et al. 1996b). Good relationships are found between the results from different genetic markers. Even if the contribution of isozymes is important by itself, molecular markers provided important clarifications for the distinction of different groups. There would be no barrier to migration of *Hevea* genes within the Amazonian basin. However, the wideness of the area and the limited dispersion of *Hevea* seeds allowed the preservation of the current structure, which is assumed to have resulted from the fragmentation of the Amazonian forest during the pleistocene period, according to the refuge theory presented by Haffer (1982). The mtDNA of Wickham clones has lesser variation since their female progenitors are restricted to a very small set of primary clones. Cytoplasmic donors for most of the improved clones are either PB 56 or Tjir 1. Obviously, this is the reason for the mtDNA profile showing only two clusters (Priyadarshan and Gonçalves 2003). A possible explanation for greater polymorphism in mtDNA of wild accessions is that many must have evolved through interspecific hybridization. The mtDNA polymorphism in wild accessions needs to be exploited fully. A molecular survey of available Amazonian accessions and isolation of competent molecular variants in their progeny are the possible exercises that can give meaningful results.

13.11.2 Gene Linkage Maps and QTLs

The construction of molecular gene linkage map in *Hevea* requires specific methodology because of high heterozygosity. Unlike annual crops, a cross between two heterozygous parents in *Hevea* can yield information up to four alleles, which are segregated further. A comprehensive genetic linkage map of *Hevea brasiliensis* has been formulated recently with the help of RFLPs, AFLPs, microsatellites and isozyme markers (Lespinasse et al. 2000a). This was accomplished through a double pseudo-test cross as per the methodology of Grattapaglia and Sederoff (1994) and a map was constituted separately for each parent. Furthermore, homologous markers segregating in both parents were ascertained and consensus map prepared. The

parents used were PB 260 (PB5/51 × PB 49) and RO 38 (F4542 × AVROS 363). F4542 is a clone of *Hevea benthamiana*. The F_1 synthetic map of 717 markers was distributed in 18 linkage groups. These comprised 301 RFLP, 388 AFLP, 18 microsatellite and 10 isozyme markers. Identification of loci was based on mobility of electrophoresis bands, necessitating verification of consistency of the location of alleles in both parental maps. The genetic length of 18 chromosomes was fairly homogeneous with an average map length per chromosome of 120 cM. Many AFLP markers were seen in clusters, which were attributed as reduced recombination frequency regions. Though the RFLP markers were well distributed all over the 18 linkage groups, these were insufficient to saturate the map. AFLPs and few microsatellites together facilitated saturating the map. However, these exercises are the initial steps for making a total genetic linkage map of *Hevea* in future. The isozymes were found to inherit following 1:1 ratio (Chevallier 1988). A partially non-random arrangement of duplicate loci observed in RFLP profiles indicates that they have homology descending from a common ancestor (Lespinasse et al. 2000a). The origin of such duplications is still unknown and *Hevea brasiliensis* continues to behave as a diploid.

As mentioned in the introduction, the upsurge of SALB looms over Asia and Africa as a potential threat for rubber plantations in future. Complete resistance to SALB was believed to be monogenic (Simmonds 1990). However, QTLs for resistance to SALB (*Microcyclus ulei*) were mapped using 195 F_1 progeny derived from a cross between PB 260 (susceptible) and RO 38 (resistant) clones (Lespinasse et al. 2000b), which was done in continuation to a genetic analysis done earlier (Seguin et al. 1996a). Eight QTLs were identified for resistance in RO 38 map through Kruskal–Wallis marker-by-marker test and interval mapping method (Lander and Botstein 1989; Oojen van et al. 1992) The F_1 consensus map confirmed results obtained in parental maps. It was further rationalized that the resistance (alleles) of RO 38 has inherited *H. benthamiana* from wild grandparent and no favorable alleles came from AVROS 363, the Wickham parent. Specificity to resistance to different strains was persistent. Two distinct forms of resistance were identified, that is, a complete resistance in the absence of sporulation lesions as in *H. benthamiana* and *H. pauciflora* and a partial resistance with a reduced rate of epidemic development (Le Guen et al. 2003). Investigations on the resistance mechanism were also conducted (Garcia et al. 1995). Le Guen et al. (2003) could detect one major QTL (*M13-lbn*) located in g 13 in RO 38 map responsible for 36–89% of phenotypic resistance. No QTL was detected for resistance against the most pathogenic isolate. Hence, a single isolate can thus completely bypass this polygenic resistance (Le Guen et al. 2007). The effect of QTL was large under natural conditions of French Guiana compared to controlled inoculation. This study should lead to marker-assisted selection (MAS), for identifying resistant genotypes with priority to geographical extent of efficiency and predictable durability. More durable resistance shall be available in other allied species and wild accessions of *Hevea* (Priyadarshan and Gonçalves 2003). However, the selection of clones having durable resistance with polygenic determinism is also important while undertaking such studies (Simmonds 1991). Darmono and Chee (1985) while studying lesion size on leaf discs identified SIAL 263, an illegitimate progeny of RRIM 501, as resistant to SALB.

13.11.3 Laticifer-Specific Gene Expression

Studies on rubber biosynthesis have gained momentum due to inquisitiveness to synthesize artificial rubber. Genes responsible for the key enzyme for polymerisation of polyisoprenes – the rubber transferase – is one of the most abundantly expressed genes in the latex. Genes expressed in the latex can be broadly categorized into three, based on their function: (a) defense genes, (b) genes for rubber synthesis, and (c) genes for allergenic proteins (Han et al. 2000). *Hevein*, a chitin-binding protein is one of the defense proteins that play a crucial role in the protection of wound sites from fungal attack. A cDNA clone (HEV 1) encoding *hevein* was isolated using polymerase chain reaction (PCR) (Broekaert et al. 1990). HEV 1 is of 1,018 base pairs and includes an open reading frame of 204 amino acids with a signal sequence of 17 amino acid residues followed by a 187 amino acid polypeptide. This polypeptide is found to contain striking features like an amino terminal region (43 amino acids) with homology to other chitin-binding proteins and amino acid termini of wound inducible proteins in potato and poplar. It was also seen that their genes were well expressed in leaves, stems and latex (Broekaert et al. 1990). Nearly 12.6% of the proteins available in the latex are defense related (Han et al. 2000).

Mainly three-rubber synthesis-related genes are expressed in the latex, namely, rubber elongation factor (REF) (Dennis and Light 1989; Goyvaerts et al. 1991), HMG CoA reductase (Chye et al. 1992) and small rubber particle protein (SRPP) (Oh et al. 1999). They constitute the 200 odd distinct polypeptides (Posch et al. 1997). The most abundantly expressed gene is REF (6.1%) and then SRPP (3.7%) (Han et al. 2000). These expressed sequences (Expressed Sequence Tags – ESTs) were compared with public databases of identified genes. About 16% of the database matched ESTs encoding rubber biosynthesis-related proteins. Analysis of ESTs revealed that rubber biosynthesis-related genes are expressed maximum followed by defense-related genes and protein-related genes (Han et al. 2000). Unlike photosynthetic genes, transcripts involved in rubber biosynthesis are 20–100 times greater in laticifers than in leaves (Kush et al. 1990). On the other hand, transcripts for chloroplastic and cytoplasmic forms of glutamine synthetase are restricted to leaves and laticifers, respectively (Kush et al. 1990), indicating thereby that the cytoplasmic form of glutamine synthetase plays a decisive role in amino acid metabolism of laticifers. Studies on laticifer specific gene expression have important implications on selection and breeding. It would be worthwhile to use transcript levels as molecular markers for early selection (Kush et al. 1990). The transcript levels of hydrolytic enzymes, namely, polygalacturonase and cellulase shall be taken as indicators for better laticifer development. It is felt that extensive studies on expression of genes are mandatory to unravel the intricacy of latex production. Detection and evaluation of more molecular markers must also help to breed *Hevea* at molecular level.

13.11.4 Direct Gene Transfer

While the in vitro plant regeneration system in rubber is getting standardized in few laboratories worldwide, efforts have been made to transform *Hevea* cells

through *Agrobacterium tumefaciens* in order to complement plant breeding efforts to increase genetic variation (Arokiaraj et al. 1990, 1994). The anther-derived calli were transformed with *A. tumefaciens* harboring *gus* and *npt*II genes encoding ß-glucuronidase and neomycin phosphotransferase, respectively. Fluorometric assay and enzyme linked immunosorbent assay (ELISA) were performed to prove the expression of *gus* and *npt*II genes respectively in calli and embryoids (Arokiaraj et al. 1996). The expression of foreign proteins in *Hevea* latex was demonstrated in 1998 (Arokiaraj et al. 1998). This transformation appeared stable even after three vegetative generations with no chimeras, indicating a single transformed plant is sufficient to have a population achieved through budding. But this exercise would not be adequate enough to take care of the stock-scion interaction and ensuing yield variation in a clonal population. Transformation of *Hevea* cells with genes for apomixis might be an alternative to circumvent stock-scion interaction. Lately, genes for human serum proteins have been expressed in rubber latex through genetic transformation (Arokiaraj et al. 2002; Yeang et al. 2002). However, the aforesaid studies have to go a long way to significantly assist breeding new clones with traits like resistance and capability to produce secondary proteins.

Another important achievement is with the expression of cDNA for farnesyl diphosphatesynthase (FPS) from a rare rubber-producing mushroom, *Lactarius chrysorrheus*, in *Escherichia coli* (Mekkriengkrai et al. 2004). Such research has to go a long way ahead to commercially produce rubber in vitro or in vivo.

13.12 Conclusions and Future Outlook

It is quite evident that rubber breeding is time consuming and labor intensive. It is here the biochemical and biotechnological tools come handy in assisting the rubber breeder in deriving and evaluating new recombinants/clones in a shorter time span possible. The foremost and essential way to shorten the period taken to derive a clone is to standardize and implement a routine biochemical/molecular marker-assisted selection system to detect high yielding accessions at the juvenile stage. Though many efforts have been incurred in this direction, a dependable method is yet to emerge out of this research. Even if a real 'Marker-Assisted Selection' applied to rubber is still to be developed and validated, so contributing to early selection, it is very probable that molecular markers, especially microsatellites will be substantially used at different levels and will improve the efficiency of rubber breeding. Another emerging area of research is metabolic profiling, that can give insights into constraints like Tapping Panel Dryness and assist in implementing an efficient early selection system. However, this does not mean that the quality of field-testing, associated with the methodology of quantitative genetics and modern statistics must be overlooked.

Rubber is traditionally propagated through bud grafting. Variations among a bud-grafted population are significant that can influence the productivity levels. As in vitro techniques are yet to make a commercial impact in rubber, a propagation

system that can circumvent the influence of stock–scion interactions needs to be achieved. One way is to derive somatic seeds that can produce true-to-mother plants. Introduction of genes for apomixis is the only way to have a homogeneous population, however, one needs to wait further since genes for apomixis are yet to be characterized in other species.

Cryo-preservation of endangered seedling trees is yet another important aspect to be looked into urgently. Since the introduction of Wickham seedlings in Asia in 1876, rubber breeding in Southeast Asia was based on Wickham material with focus on yield improvement, while research in Latin America was devoted to create *Microcyclus*-tolerant and productive clones. This leads to two constant parallel ways of achieving clones suited to the needs of respective regions. Since *Microcyclus* is a threat to Southeast Asian countries, international multi-location experiments must be given priority.

As a perennial crop, rubber breeding has been influenced by the grafting technique, which permitted the development and multiplication of clones evolved from recombinants. In spite of the implementation of early selection techniques, rubber breeding is impeded by the time-length of the selection process, and the limited creation of full-sib families gained through low success rates of hand-pollination. Standardization of a rapid early selection technique is the immediate requisite.

In order to broaden the genetic base, varied attempts were made for introducing new germplasm to Asia, including species allied to *H. brasiliensis*, among which the international IRRDB collection was the most significant. But, due to low yield level of this germplasm and to the length of the breeding process, benefits will be distributed only over a long term. Apart from the creation of new clones for development, this research requires specific germplasm pre-breeding programs to produce new parents for recombination breeding prior to selection. The spectrum of useful genetic variation need to be enlarged, especially through utilizing variable cytoplasmic donors, since most oriental clones received cytoplasm either from PB 56 (through PB 5/51) or from Tjir 1. Biotechnological tools should assist in finding useful variation among the wild germplasm and allied species, especially cytoplasmic genetic diversity and QTLs for resistance to diseases.

There must be an effort to split Wickham population into different groups at the molecular level. This will pave the way delimiting the development of many related clones and of inbreeding depression. Also, with regard to rubber cropping in relation to overall economy (new locations, new objectives, and new economic constraints), rubber breeding needs to address derivation of larger scope of clones adapted to varied biotic or abiotic stresses, and to varied specifications including rubber quality. Consequently, it would be required not only to select elite clones but also to describe the behavior of a larger range of clones at small-scale experimental level and in different environments (development of clonal databases). There are newer clones derived by fast developing economies like China, Vietnam and Thailand. All these and the existing popular clones shall be enlisted in the clonal database, probably under the umbrella of IRRDB. Such databases should provide details of not only clones but also judicious tapping schedules and discriminatory fertilizer doses for new locations. Such a description will also help to suggest better arguments for the

diversification of recommended clones. More emphasis on eco-physiology research could provide the necessary results for achieving some or all of those goals.

Research devoted to SALB resistance involving recurrent backcrossing of Amazonian-resistant clones (mainly the *H. benthamiana* F 4542 or derived clones) with Wickham high-yielding clones to evolve different resistance sources (clone or polyclonal seedling population) needs to be augmented. This strategy could also be applied to specific programs aimed at selecting clones resistant to *Corynespora*, or *Oidium*, or other diseases within integrated approaches.

As rubber wood has become an ancillary source of income, rubber breeding now has to integrate new traits, especially traits based on architecture and on biomass production, in order to produce better-optimized 'latex-timber' clones.

Although in vitro culture still meets different obstacles, somatic embryogenesis is the gateway for the implementation of targeted genetic transfers, so accelerating genetic progress on agricultural traits or widening the scope such as the possible production of proteins in the latex.

International cooperation and the interface between research institutions and product transforming private sector must be promoted in order to amplify the efficiency and efforts of rubber breeders. Even if the needs of smallholders are addressed with priority, industrial estates have provided a significant contribution in land facilities and logistics for large-scale and long-term testing of clones, and would continue to do it. With many projects toward this direction, IRRDB can play a key role in this field of cooperation in rubber breeding.

Small holdings are the backbone of rubber economy. With fluctuations in domestic and international prices, it is highly essential to train the farmers on feasible investment procedures. This aspect should occupy a significant place in the participatory breeding programs that can immensely help the farmers to educate and innovate themselves.

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Part IV
Beverages

Chapter 14
Breeding Coffee (*Coffea arabica*) for Sustainable Production

Lashermes Philippe, Bertrand Benoît, and Etienne Hervé

14.1 Introduction

Coffee is one of the world's most valuable export commodities, ranking second in the world market after petroleum products. The total retail sales value exceeded US$70 billion in 2003 and about 125 million people depend on coffee for their livelihoods in Latin America, Africa and Asia (Osorio 2002). Commercial production relies on two species, *Coffea arabica* L. and *C. canephora* Pierre. The cup quality (low caffeine content and fine aroma) of *C. arabica* makes it by far the most important species, representing 70% of the world production.

C. arabica has its primary centre of genetic diversity in the highlands of southwest Ethiopia and the Boma Plateau of Sudan. Wild populations of *C. arabica* have been also reported in Mount Imatong (Sudan) and Mount Marsabit (Kenya) (Thomas 1942, Anthony et al. 1987). Cultivation of *C. arabica* started in southwestern Ethiopia about 1,500 years ago (Wellman 1961). Modern coffee cultivars are derived from two base populations – known as Typica and Bourbon – which were spread worldwide in the eighteenth century (Krug et al. 1939). Historical data indicate that these populations were composed of progenies of very few plants, that is, only one for the Typica population (Chevalier and Dagron 1928) and the few plants that were introduced to the Bourbon Island (now Reunion) in 1715 and 1718 for the Bourbon population (Haarer 1956). Breeders exploited these narrow genetic bases, resulting in Typica- and Bourbon-derived cultivars with homogeneous agronomic behaviour characterised by high susceptibility to many pests and low adaptability (Bertrand et al. 1999).

As with cereal crops, Arabica coffee growing was turned completely upside down by a veritable green revolution over the 1970–1990 period. However, as it was not a matter of solving the world's hunger problems, which was the case for rice, wheat or maize (Borlaug 1968), the Arabica revolution received little media attention. Traditionally, coffee was grown under forest trees or planted trees (often used for firewood). Use of traditional varieties (Bourbon, Typica) with a tall growth habit, or

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even wild types (Ethiopia), with planting densities of around 1,500–3,500 trees/ha was commonplace. Fertiliser use in such a system was very limited (0–100 units of nitrogen). Through several concurrent technical advances in 1950s and 1960s that cropping system evolved and the conditions for a veritable green revolution were created. From an extensive system under forest cover, a switch was made to full sunlight with abundant use of fertilisers (notably 300–600 units of nitrogen/ha and 150–800 units of potassium) and pesticides. As a result, yields more than doubled from 150 to 500 kg of green coffee/ha to more than 1,000 kg, and even exceptional yields of more than 3,500 kg/ha under good ecological conditions. Just as the new varieties of wheat were at the heart of the green revolution, the new semi-dwarf mutant varieties (that is Caturra and Catuai derived from the traditional Bourbon and Typica varieties; Carvalho 1988) served as a catalyst (Castillo 1990) in this coffee-growing revolution. By using those varieties, it was possible to double or even treble planting densities (5,000–8,000 trees/ha).

The plantation sector moves into the new millennium and the coffee industry has several new challenges to address. The coffee production is surplus world-wide and the prices are highly variable and frequently low. Hence, minimising the production cost and maintenance of product quality to suit consumer preferences are the key factors to thrive in highly competitive international market. The new wave of sustainable development and the reality of World Trade Organization (WTO) have led to new quality trends in commodity sector. Accordingly, stringent environmental/eco-friendly quality parameters are being imposed in producing countries. These developments have direct impact on R&D and there is a need to reorient the research priorities to address the new challenges. Genetic improvement for sustainable production appears therefore as an essential issue. In this chapter, we review research aspects linked to the origin and genetic diversity of *C. Arabica*, the introgressive breeding, the development and micropropagation of improved F_1 hybrid varieties.

14.2 Genetic Resources

14.2.1 Phylogeny of Coffea Species

Coffee trees are classified into two genera, *Coffea* and *Psilanthus*, each genus being divided into two subgenera (Bridson and Verdcourt 1988). All *Coffea* species are native to the inter-tropical forest of Africa, Madagascar and islands of the Indian Ocean (Mascareign and Comoro Islands), while species belonging to the genus *Psilanthus* originate from either Asia or Africa. The subgenus *Coffea* encompasses more than 80 taxa so far identified, including the two species of economic importance: *C. arabica* L. and *C. canephora* Pierre (Charrier and Berthaud 1985). *Coffea* species are diploid ($2n = 2x = 22$), except *C. arabica* ($2n = 4x = 44$), which is self-fertile and allotetraploid (Carvalho 1952). Molecular phylogeny of *Coffea* species has been established based on DNA sequence data. The internal transcribed

spacer (ITS 2) region of the nuclear ribosomal DNA (Lashermes et al. 1997) as well as the chloroplast DNA variation (Cros et al. 1998) were successfully used to infer phylogenetic relationships of *Coffea* species (Fig. 14.1). The results suggest a radial mode of speciation and a recent origin in Africa for the genus *Coffea*. Several major clades were identified, which present a strong geographical correspondence (that is West Africa, Central Africa, East Africa and Madagascar).

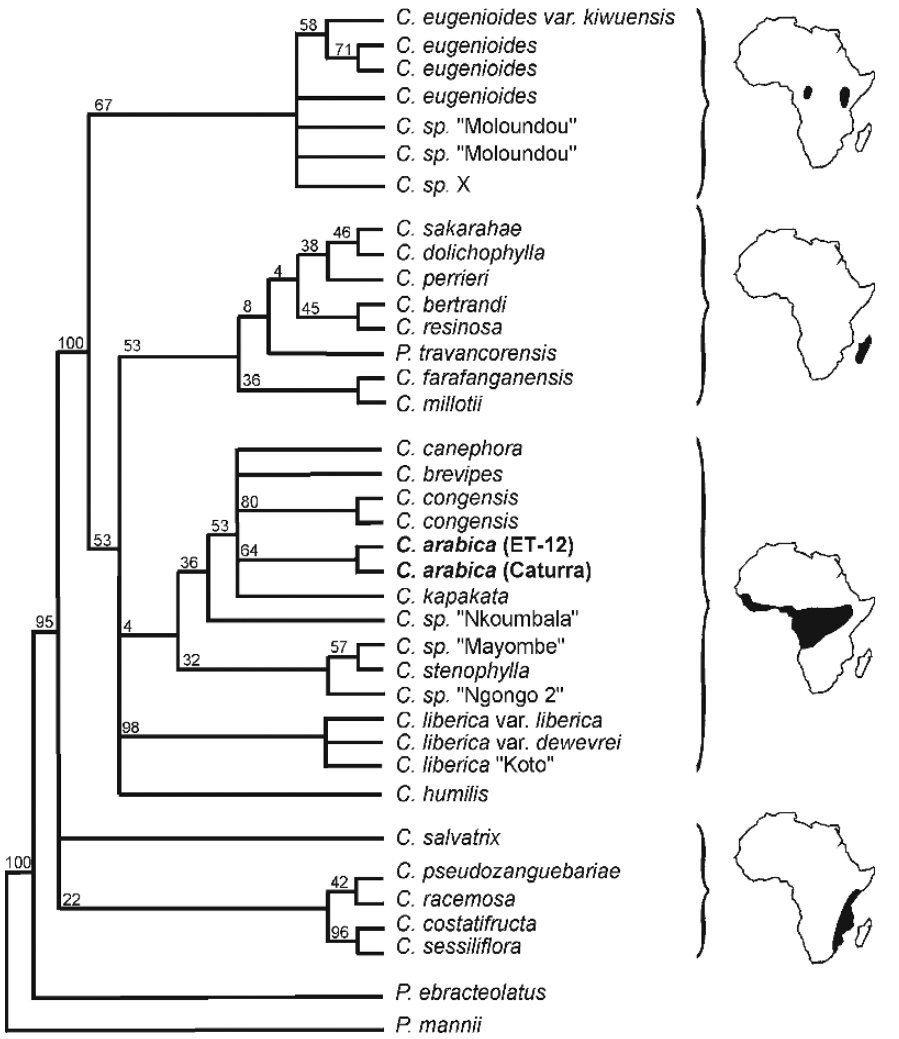

Fig. 14.1 Molecular phylogeny of Coffea species. Strict consensus of Wagner tree resulting from parsimony analysis of ITS 2 (nuclear ribosomal DNA) sequence data. Numbers above the nodes represent bootstrap values (%). Geographical distributions of the major groups are also indicated (Lashermes et al. 1997)

14.2.2 Origin and Genome Structure of *C. arabica*

The genome constitution and mode of speciation of *C. arabica* have been subjected to several investigations. Restriction of fragment length polymorphism (RFLP) loci-markers in combination with genomic in situ hybridisation (GISH) were used to investigate the origin of *C. arabica* (Lashermes et al. 1999). By comparing the RFLP patterns of potential diploid progenitor species with those of *C. arabica*, the source of the two sets of chromosomes or genomes, combined in *C. arabica* was specified.

The genome organisation of *C. arabica* was confirmed by GISH using simultaneously labelled total genomic DNA from the two putative genome donor species as probes (Raina et al. 1998, Lashermes et al. 1999). These results clearly suggested that *C. arabica* is an amphidiploid (that is C^aE^a genomes) resulting from the hybridisation between *C. eugenioides* (E genome) and *C. canephora* (C genome) or ecotypes related to those diploid species. Results also indicate low divergence between the two constitutive genomes of *C. arabica* and those of its progenitor species, suggesting that the speciation of *C. arabica* took place very recently. Precise localisation in central Africa of the speciation process of *C. arabica* based on the present distribution of the coffee species appeared difficult since the constitution and extent of tropical forest varied considerably during the late quaternary period. Furthermore, investigations suggest that homoeologous chromosomes do not pair in *C. arabica*, not as a consequence of structural differentiation, but because of the functioning of pairing regulating factors (Lashermes et al. 2000a).

14.2.3 Genetic Diversity

A whole range of different techniques has been used to detect polymorphism at the DNA level, including randomly amplified polymorphic DNA, amplified fragment length polymorphism and simple sequence repeats or microsatellite (Anthony et al. 2002, Combes et al. 2000, Lashermes et al. 1996, 2000b, Mettulio et al. 1999). The use of molecular methods has opened up new possibilities for genetic analysis and provides new tools for the efficient conservation and use of coffee genetic resources. For instance, the genetic diversity in *C. arabica* appeared extremely reduced in comparison to the diversity observed in *C. canephora*. This low genetic diversity has been attributed to the allotetraploid origin, reproductive biology and evolution process of *C. arabica* (Lashermes et al. 1996). The base populations of cultivated coffee clearly originated from wild coffee collected in southwestern Ethiopia (Fig. 14.2). The genetic differentiation was low between the Typica and Bourbon groups, but much higher between wild and cultivated coffee. Spontaneous accessions collected in the primary centre of diversity appeared to constitute a valuable gene reservoir. These results should increase interest in wild coffee for the purpose of broadening the genetic base of cultivars.

Transfer of desirable genes in particular for disease resistance from diploid species like *C. canephora* and *C. liberica* into tetraploid Arabica cultivars without affecting quality traits has been the main objective of Arabica breeding (Carvalho

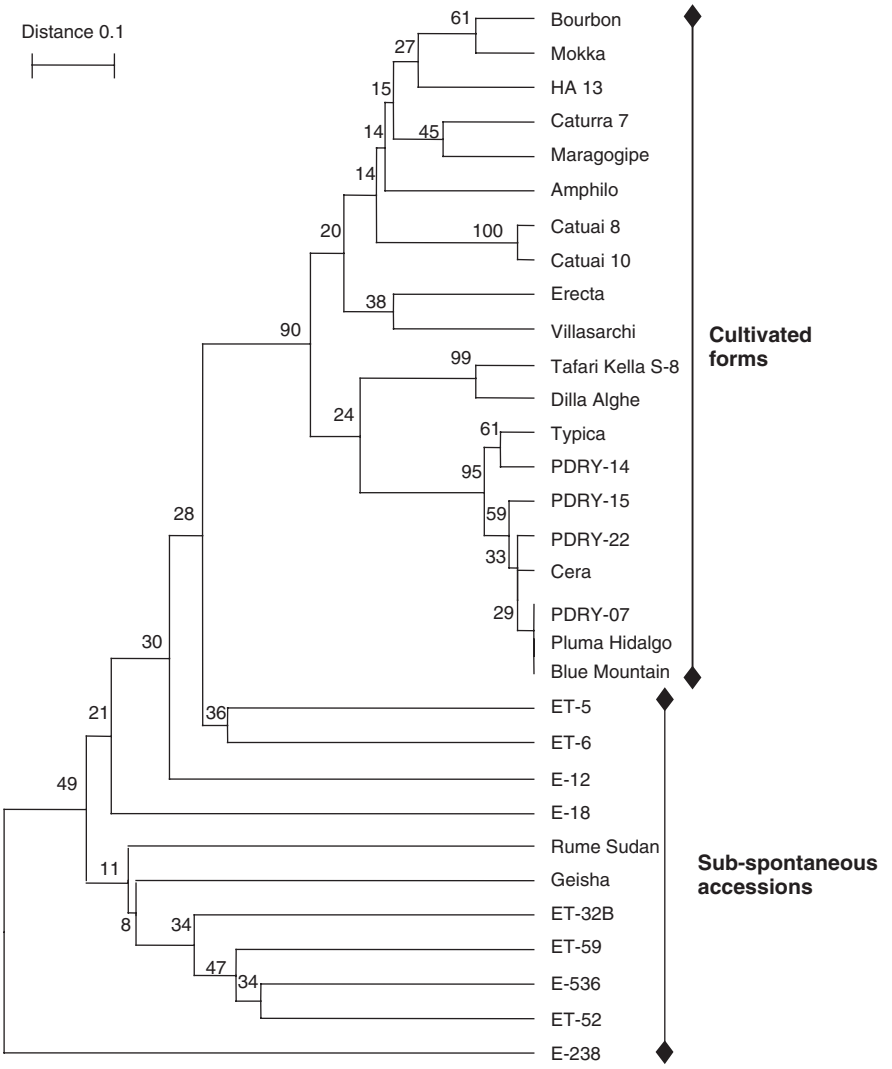

Fig. 14.2 Diversity analysis. Dendrogram of the C. arabica accessions generated by group average clustering (UPGMA) using AFLP-based genetic distance. Numbers below branches are bootstrap values (%)

1988, Van der Vossen 2001). To date, *C. canephora* provides the main source of disease and pest resistance traits not found in *C. arabica*, including coffee leaf rust (*Hemileia vastatrix*), Coffee Berry Disease (*Colletotrichum kahawae*) and root-knot nematode (*Meloidogyne spp.*). Similarly, other diploid species present considerable interests in this respect. For instance, *C. liberica* has been used as source of resistance to leaf rust (Srinivasan and Narasimhaswamy 1975) while *C. racemosa* constitutes a promising source of resistance to the coffee leaf miner (Guerrero Filho et al. 1999).

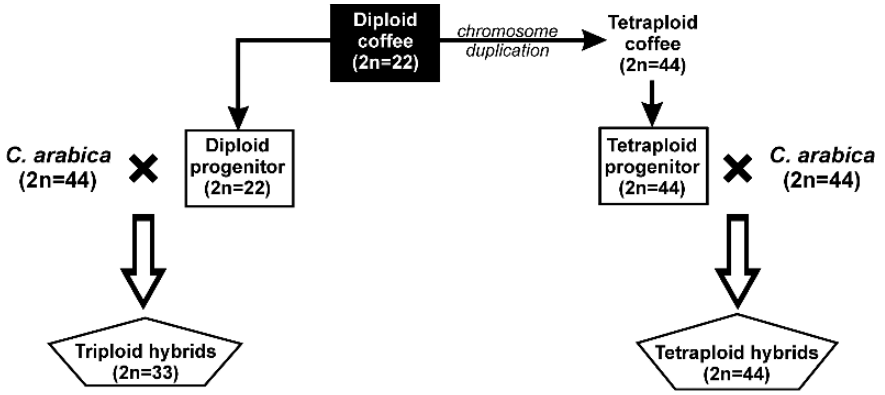

Fig. 14.3 Triploid and tetraploid strategies of gene introgression from diploid coffee species into C. arabica genome

Exploitation of coffee genetic resources has so far relied on conventional procedures in which a hybrid is produced between an outstanding variety and a donor genotype carrying the trait of interest, and the progeny is backcrossed to the recurrent parent. Undesirable genes from the donor parent are gradually eliminated by selection. In so doing, conventional coffee breeding methodology faces considerable difficulties. In particular, strong limitations are due to the long generation time of coffee tree (5 years), the high cost of field trial and the lack of accuracy of current strategy. A minimum of 25 years after hybridisation is required to restore the genetic background of the recipient cultivar and there by ensure good quality of the improved variety. Genes from diploid coffee could be transferred into the *C. arabica* genome through the production of triploid ($2n = 33$) or tetraploid ($2n = 44$) interspecific hybrids (Fig. 14.3). Triploid hybrids are derived from a direct cross between the diploid progenitor and *C. arabica* while tetraploid hybrids are obtained by crossing both species after chromosome duplication of the diploid coffee. The use of triploid hybrids is more difficult than tetraploid hybrids because of low fertility (Krug and Mendes 1940, Berthaud 1978a,b). However, high producing seedlings have been selected as early as the first backcross to *C. arabica* or after the second generation by selfing (Orozco Castillo 1989).

14.3 Molecular Analysis

14.3.1 Introgression Assessment

The presence of *C. canephora* DNA fragments was sought in accessions derived from a tetraploid interspecific hybrid known as Timor Hybrid. The Timor Hybrid originated from a spontaneous cross between *C. arabica* and *C. canephora* on the island of Timor (Bettencourt 1973). Following a backcross with a *C. arabica* cultivar

(that is cvs. Caturra or Villasarchi), progenies were selfed and selected over three to five generations in several important coffee-producing countries such as Brazil, Colombia and Kenya. Although varying between Timor Hybrid-derived genotypes, the amount of alien genetic material appeared substantial (Lashermes et al. 2000b). Furthermore, the incidence of *C. canephora* gene introgression on biochemical composition of beans and cup quality in Arabica coffee breeding lines was investigated (Bertrand et al. 2001, 2003, 2005a). High amount of introgression appeared frequently associated with lower quality factors. However, lines combining resistance to leaf rust and root-knot nematode (*M. exigua*) and good cup quality were successfully developed.

A similar study was undertaken to analyse the S.288 progeny of a putative natural hybrid ($C.\ arabica \times C.\ liberica$) and accessions (F2 and F4) derived from the cross (S.288 × cv. Kent) (Prakash et al. 2002). The number of introgression markers was found to be similar in the *C. liberica* introgressed accessions and in the *C. canephora* introgressed accessions. Analysis of genetic relationships in the introgressed lines suggested that introgression was limited to few fragments. Moreover, the alien genetic material appeared to be fixed and there was no elimination or counter-selection over generations, from introgressed parent to F_4.

14.3.2 Strategy

14.3.2.1 Factors Controlling Gene Introgression

During the last few years, the behaviour of interspecific hybrids between *C. arabica* and the diploid species, *C. canephora* and *C. eugenioides*, has been investigated. Numerous plant populations resulting from the backcross (BC_1) of either triploid or tetraploid interspecific hybrids to *C. arabica* were analysed (Herrera et al. 2002a,b, 2004). Flow cytometric analysis of the nuclear DNA content revealed that most of the BC1 individuals were tetraploid or nearly tetraploid, suggesting that among the gametes produced by the interspecific hybrids, those presenting 22 chromosomes were strongly favoured. Furthermore, molecular markers (that is RFLP, microsatellite and AFLP) combined with evaluation of morphological characteristics and resistance to leaf rust were applied to verify the occurrence of gene transfer from the donor species into *C. arabica*, and to estimate the amount of introgression present in BC_1 individuals. While a high amount of introgression was observed in the progenies derived from the tetraploid interspecific hybrids, the BC_1 individuals generated from the triploid interspecific hybrids exhibited contrasted situations. The mean proportion of introgressed markers per plant was significantly lower in populations derived from *C. eugenioides* than from *C. canephora*. Moreover, the comparison of reciprocal progenies between *C. arabica* and triploid interspecific hybrids ($C.\ arabica \times C.\ canephora$) used as male or female parent revealed a very strong effect of the backcross direction. A severe reduction in frequency of *C. canephora* introgressed markers was observed when the triploid hybrids were

used as the male parent. Breeding strategies based on gene introgression can now be designed according to the objectives of selection.

14.4 Development of F1 Hybrid Varieties

Since the 1980s, several researchers have proposed the creation of hybrid varieties to help in increasing genetic diversity, notably by using wild Sudan–Ethiopian origins (Charrier 1978) and to exploit heterosis between genetic groups (Walyaro 1983, Van der Vossen 1985). Sudan–Ethiopian origins provide resistance to nematodes (Anzueto et al. 2001), partial resistance to leaf rust (Gil et al. 1990) and resistance to CBD (Bellachew 2001) and likely is of a better beverage quality. As regards heterosis in the species, Carvalho and Monaco (1969), Walyaro (1983), Ameha (1990), Bellachew (1997), then Cilas et al. (1998) demonstrated its existence by intercrossing. In most self-fertilizing species, hybridisation comes up against the difficulty of mass reproduction of heterozygous structures. This is not the case with the *C. arabica* species, as several technical solutions can overcome this difficulty. Somatic embryogenesis appears as the most promising technique (see Part 14.5.1). However, both large-scale rooting of cuttings and hybrid seed production by controlled pollination are feasible.

14.4.1 Description of the Hybrid Vigour in Coffea arabica

In *C. arabica*, heterosis calculated on the basis of the best parent was evaluated from crosses between different genetic pools (Table 14.1). The heterosis observed by different authors varies from 10 to 40% (Ameha 1990, Carvalho and Monaco 1969, Fazuoli et al. 1993, Walyaro 1983, Netto et al. 1993), with the notable exception of the heterosis reported by Cilas et al., 1998, which reached over 200%. The heterosis found in Central America (22.0 to 47.0%) was globally around the same magnitude as that observed by the majority of authors. The results obtained by Cilas et al. (1998) can be explained, in our opinion, by the poor performance of the parental lines under the difficult climatic and soils conditions of Cameroon.

For the vegetative variables, significant differences appeared between the two populations (hybrids and lines) for the length of primaries and for stem girth (Bellachew 2001, Bertrand et al. 2005b). Otherwise, the hybrids were not significantly different from the lines for plant height, number of primary branches, internode's length of primaries, leaf area. In Central America or in Ethiopia, the yield differences between the parental lines and the hybrids were not explained by the yield components, such as the number of fruits per node or by the weight of 100 beans, which were identical for both populations. Finally, the F1 hybrid population showed lower fertility than the population of lines. In Central American conditions, the difference in fertility rate was from 1.2 to 6.3% for floating fruits. In coffee, the number of seeds per fruit depends on ovule fertility (Louarn 1992). Neither could

Table 14.1 Heterosis values estimated in different genepools

Genetic pools	Author (year)	Heterosis for yield (%), [min, max], (density/ha)
A/INTRA-POOL	Ameha (1990)	17, [–8, 60]
'Wild' × 'Wild'*	Bellachew (2001)	57, [9, 91]
'Wild' × 'Wild'	Cilas et al. (1999)	> 200
'Bourbon' × 'Bourbon'	Carvalho and Monaco (1969)	10
	Fazuoli et al. (1993)	25
'L D HdT' × 'L D HdT'	Bertrand 2000	14.7 [−3, 21] (7,086 a/ha)
B/INTER-POOL		
'Bourbon' × L D HdT	Santacreo (pers. com., 1992)	35
'Bourbon' × L D HdT,	Walyaro (1983)	25; [10, 209]; (3,333 or 6,667 a/ha)
'Bourbon × Bourbon', 'Bourbon' × 'Wild', L D HdT × 'Wild'		
'Bourbon' × L D HdT	Netto et al. (1993)	40
'Bourbon' × 'Wild'	Bertrand et al. (2005b)	25; [19–28]; (3,333 or 5,000 a/ha)
L D HdT × 'Wild'	Bertrand (unpublished datas)	37; [5–70]; (3,333 or 5,000 a/ha)

*'Bourbon' = Caturra, Catuai, Mundo Novo, Bourbon; 'L D HdT' = lines derived from the Timor hybrid; 'Wild' = accessions from Ethiopia or Sudan.

heterosis be explained by better fertility, since hybrid fertility was even lower than that of the lines. Heterosis seemed to be permanently reflected in longer primary branches. This difference in length, which could not be attributed to a difference in internode's length, came from a larger number of internodes formed over the same time lapse.

The hybrid vigour in the Arabica species needs to be considered in relation to those obtained with other self-fertilising species. In rapeseed, Lefort-Buson and Dattée (1985) found heterosis of 12.0–18.0% between populations of different origin. In barley, heterosis under suitable growing conditions was found to be between 10.0 and 25.0% (Scholz and Kunzel 1987). In wheat, heterosis was 17–40% depending on density (Oury et al. 1990). The results obtained with *C. Arabica*, therefore showed heterosis that was comparable with that of most self-fertilizing species. The increase in biomass has led many authors to study heterosis in relation to density (Orozco 1975). The hypothesis is that competition between plants might lessen heterosis. However, the first results obtained from a network of trials set up in Central America, under commercial planting density conditions (5,000 to 10,000 plants/ha), do not seem to reveal such a tendency. Though more vigorous, hybrids seem to withstand strong competition very well. In one hybrid trial, at a density of 7,000 trees/ha, the difference between the best hybrid and the best line amounted to 36% (over four harvests). Heterosis would seem to go hand-in-hand with greater homeostasis, which enables hybrids to withstand stresses due to strong competition.

14.4.2 Performance of Selected Hybrids

In Central America, multi-trait selection in a hybrid population led to substantial expected genetic progress for yield and low progress for the weight of 100 beans (Bertrand et al. 2005b). On the other hand, selection for productivity and fertility were opposite. This means that it is difficult to select for yield without, in return, reducing fertility. Conversely, by selecting for better fertility, no genetic progress will be made for yield within the population of hybrids. We put forward the hypothesis that heterozygosity leads to a drop in fertility and an increase in vigour. Indeed, it has been found that pedigree selection always goes hand in hand with progress in fertility (without directed selection). Conversely, 'Arabusta' trees, which are interspecific hybrids obtained between *C. arabica* and *C. canephora* display a very high level of hybrid vigour combined with very high sterility (Reffye 1973). The most productive hybrids came from introgressed maternal lines derived from the Timor Hybrid crossed by wild origins (Table 14.2).

14.4.3 F1 Hybrid and Coffee Beverage Quality

The organoleptic evaluations under various edapho-climatic conditions and elevations, by comparing F1 hybrids with traditional cultivars ('Bourbon') for bean chemical contents and cup quality did not show any clear difference (Bertrand et al. 2006). F1 hybrids appeared in turn to be inferior, similar or superior to traditional cultivars for certain attributes, such as acidity or aroma. Regarding the overall standard, F1 hybrids were equivalent or superior to traditional cultivars. For caffeine, as for trigonelline, the hybrids did not differ from the traditional varieties. The hybrids showed a tendency to be slightly richer in CGA than the traditional varieties. On the other hand, elevation did not seem to influence fat contents for the F1 hybrids. These new varieties might produce 30–70% more than traditional varieties, and were

Table 14.2 Performances of a set of hybrids selected in two trials in Central America, compared to the two highest yielding lines in the trial for the target traits. Means followed by the same letter are not significantly different at the 0.05 level (Newman–Keuls tests)

Trials	Type of genotype	Genotype	YIELD (g)	DWMEAN** (g)	FFMEAN*** (%)
Trial 1	Line	A	9,871 c	17.93 b	2.92 b
	Line	B	1,2954 b	18.15 b	2.37 b
	Hybrid	B × W1	1,4430 ab	19.67 a	5.78 a
	Hybrid	B × W2	1,6316 a	20.01 a	2.14 b
Trial 2	Line	C	11 595 c	16.82 c	6.5 a
	Line	D	13 802 bc	18.88 ab	5.3 a
	Hybrid	E × W3	20 291 a	19.63 a	6.1a
	Hybrid	F × W4	20 219 a	18.86 ab	5.3 a
	Hybrid	E × W5	18 941 a	18.23 b	4.8 a
	Hybrid	F × W6	15 345 b	17.00 c	4.3 a

*Yield = recorded for 5 years; **DWMEAN = dry weight of 100 beans; ***FFMEAN = fraction of berries with one empty seed cavity (Bertrand et al. 2005b).

exceptionally vigorous. Higher vigour resulted in better nutrient supply to the fruits, whatever the elevation. The use of F1 hybrids should thus contribute to reducing variation in the fat content of coffee beans, and at the same time reduce variations in beverage quality.

14.5 Mass In Vitro Propagation Through Somatic Embryogenesis

Horticultural vegetative propagation is still not available for *C. arabica*, probably due to the greater difficulty in achieving satisfactory multiplication rates (Van der Vossen 1985). Moreover, the difficulty to transport the cuttings and the risk of disease propagation has prohibited the use of cuttings at the commercial level. Similarly, male sterility is still not available for the propagation of heterozygous materials. Arabica varieties are sold in seed form after a relatively lengthy pedigree selection process, taking at least 20 years. Micropropagation techniques may be applicable for mass production of selected *C. canephora* clones, and for interspecific hybrids, such as Arabusta, but they are of particular interest in the case of F1 hybrids of *C. arabica*. Among these techniques, somatic embryogenesis has the greatest multiplication potential, which enables numerous technical simplifications, and should consequently entail the lowest production costs (Etienne et al. 1999, Berthouly and Etienne 1999).

14.5.1 Somatic Embryogenesis

Somatic embryogenesis in several *Coffea* species and genotypes is well documented (Starisky 1970, Söndahl and Sharp 1977, Pierson et al. 1983, Dublin 1984, Yasuda et al. 1985, Berthouly and Michaux-Ferrière 1996, Berthouly and Etienne 1999). Among the different processes, which have been described using leaf sections as explants, the high frequency somatic embryogenesis is the most adapted for large-scale propagation. Two media are commonly used: an induction medium for primary callogenesis, and a secondary regeneration medium to produce embryogenic friable callus regenerating several hundred thousand somatic embryos per gram of callus (Söndahl and Sharp 1977, Dublin 1984, Berthouly and Michaux-Ferrière 1996, Etienne 2005). The high-frequency procedure (from leaf explants to somatic embryos) takes about 7–8 months for *C. canephora* and Arabusta, and 9–10 months for *C. arabica*. This process enables the use of a liquid medium for both embryogenic tissue proliferation, and the regeneration phase, and it was consequently preferred for scale-up and development of mass propagation procedures.

14.5.1.1 Mass Somatic Embryo Production

In the two cultivated species, *C. canephora* and *C. arabica*, mass somatic embryo production has been successfully carried out in liquid medium (Starisky and

Van Hasselt 1980, Zamarripa et al. 1991, Ducos et al. 1993, Van Boxtel and Berthouly 1996). From 1 g of embryogenic tissue, Zamarripa et al. (1991) obtained high rates of 200,000 *C. canephora* somatic embryos in Erlenmeyer flasks, and Ducos et al. (1993) obtained 600,000 embryos/l medium in a bioreactor. With *C. arabica*, lower yields of embryos were obtained under such conditions compared with *C. canephora* and Arabusta. Zamarripa et al. (1991) and Zamarripa (1993) reported yields of about 46,000 embryos/3l Erlenmeyer flask or 20,000 embryos/l in a bioreactor.

Problems with somatic embryo quality (morphological abnormalities, hyperhydricity, asynchronous development and size heterogeneity) and the difficulty in extending embryo development beyond its torpedo stage in liquid medium have been reported in most of these studies. The frequency of embryos showing normal torpedo morphology varied from 7 to 20% and those embryos were then selected by hand and subcultured frequently for further in vitro germination, resulting in plant conversion rates varying from 30 to 60%. These laborious manipulations greatly increase production cost, which explains why somatic embryogenesis has never been applied on a commercial scale. Moreover, the elevated cost of conventional bioreactors prohibited their use when propagating several varieties. A somatic embryogenesis procedure using a temporary immersion bioreactor was developed for *C. arabica* F1 hybrids, enabling mass and virtually synchronous production of mature somatic embryos, without the need for selection before acclimatisation (Etienne et al. 1997, Etienne-Barry et al. 1999, Fig. 14.4A). Depending on the

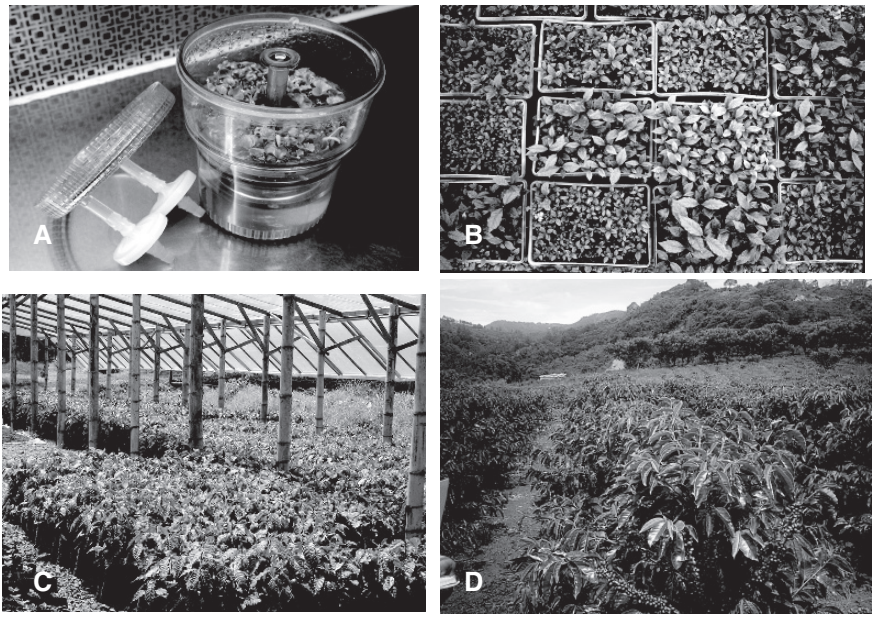

Fig. 14.4 Somatic embryogenesis in coffee

genotypes, yields ranging from 15,000 to 50,000 somatic embryos per gram of embryogenic suspension cell mass were recorded. The proportion of normal torpedo type embryos produced by temporary immersion is usually ≥ 90%. Moreover, using short immersion times (1 min) overcame hyperhydricity (Etienne and Berthouly 2001, Albarrán et al. 2005).

14.5.1.2 Direct Sowing of Somatic Embryos in the Nursery

Conditions for direct sowing of *C. arabica* somatic embryos produced in a temporary immersion bioreactor on horticultural soil have also been developed (Etienne-Barry et al. 1999). Plant conversion rates for mature somatic embryos, that is, possessing a pair of open cotyledons, along with a well-developed chlorophyllous embryonic axis, frequently reach 70% in the nursery (Fig. 14.4B). It was shown that 86% of embryos in the same 1l-RITA® bioreactor reached the 'mature' stage and could be directly sown (Barry-Etienne et al. 2002). However, they revealed morphological heterogeneity in terms of cotyledon area, which resulted in heterogeneity in the nursery, mainly related to retarded growth in plantlets derived from somatic embryos with small cotyledons. Direct sowing reduces handling time to 13% and shelving area requirements to 6.3% of the values obtained with conventional acclimatisation of plants developed on semi-solid media (Etienne-Barry et al. 1999). Moreover, a physiological approach has recently shown that even though the plantlets obtained with direct sowing were still small compared with seedlings (ratio 1:3), they were readily acclimatised to nursery conditions and had vigorous aerial and root systems and active growth (Barry-Etienne et al. 2002). The economic viability of direct sowing was then proved along with the quality of the regenerated plantlets.

14.5.1.3 Field Performance and Trueness to Type

Few data are available on the field performance of coffee plants produced by somatic embryogenesis, though such information is critical. Ducos et al. (2003) reported that there was no somaclonal variation in 5,067 trees originating from 5- to 7-month-old embryogenic cell suspensions of five *C. canephora* clones. We observed the same genetic stability of *C. canephora* in the 18,000 parents of the Nemaya variety planted in field gardens (Bertrand et al. 2002). On the other hand, somaclonal variation has frequently been found in *C. arabica* and must be taken into account for commercial development of somatic embryogenesis. Vegetative characteristics, productivity, fertility and the bean biochemical, mineral and organoleptic characteristics of *C. arabica* F1 hybrids derived from a 5-month-old embryogenic suspension were identical to those in a microcutting control (Etienne and Berthouly 2001). In this study, the off-type frequency was 2.1%. Since this work, we have evaluated 60,000 other plants in the field belonging to 20 clones of *C. arabica* F1 hybrids. We now estimate that the risk of somaclonal variation is between 1 and 5%, depending on the genotype. The off-type percentage drastically increases when embryogenic suspensions proliferate beyond the sixth month (Etienne and

Bertrand 2003). Cryopreservation of young coffee embryogenic suspensions may be routinely used to reduce the risk of somaclonal variation. The most frequent off-types are angustifolia trees (Fig. 14.4D), which might be aneuploid ($2n = 41$, for example). Dwarf trees, giant trees, variegata trees and trees exhibiting a change in the colour of immature leaves were also characterised. Apart from the dwarf type, the majority of off-types can be detected and eliminated in the nursery.

14.5.2 Mass Propagation of C. arabica F1 Hybrids

F1 hybrids obtained from crosses between cultivated varieties and semi-wild trees originating in Ethiopia or Sudan led to 30% higher productivity in Central America, along with resistance to leaf rust and nematodes, whilst maintaining an excellent cup quality (Bertrand et al. 2005b). In Tanzania, 12 more or less complex crosses between commercial lines and parents, such as Rume Sudan and HdT, were selected for their resistance to leaf rust, or CBD, or both. High frequency somatic embryogenesis was chosen for large-scale evaluation of these heterozygous structures and subsequent commercial dissemination. Embryo mass production techniques involving temporary immersion bioreactors and direct sowing of embryos in the nursery have been successfully used in the last 6 years for propagation and large-scale evaluation of F1 selected hybrids. A large-scale multi-site trial totalling, to date, 100,000 trees from 20 selected hybrids has been set up in five Central American countries, supervised by the national coffee research institutes. A similar field trial including 18,000 selected hybrids multiplied by somatic embryogenesis was also established by TACRI in Tanzania.

The different pilot productions of arabica F1 hybrids in various countries represent a first step towards a commercial production in the short term. Productions of millions plants in industrial units were undertaken by some companies. The purpose of current research on the procedure is primarily to reduce production costs in order to be competitive with plants traditionally produced from seed and sold for less (USD 0.10–0.30/plant). In addition, as planting densities are very high for *C. arabica* dwarf varieties (between 5,000 and 10,000 trees/ha) planting costs are substantial. To achieve this objective, research is focusing on optimising the acclimatisation/nursery phases and on improving the efficiency of various stages in the procedure, facilitated by a scale-up.

14.6 Conclusions and Perspectives

Trees are the cornerstone of natural biodiversity and the best protectors of soil fertility. Consequently, in the case of coffee growing, a return to shaded cultivation is called for. In the face of global warming, there is pressure to reforest coffee plantations. In an agroforestry system, trees recycle organic matter and reduce effluent loss. The right conditions for reforestation exist in most producing countries.

Most of the stakeholders in the world are considering a return to shaded coffee growing through agroforestry systems, which could improve coffee farmers' incomes in the long term through diversification (timber production), through the production and marketing of better quality coffee and, eventually, through the payment of incentives for environmental services provided by these ecologically sound coffee systems. Unfortunately, switching from a full sunlight system to a shaded system is slow and expensive. Installing shade reduces yields by 20–30% (Vaast et al. 2005). We feel that rational use of new hybrid varieties with a high yield and quality potential should act as a catalyst in increasing the sustainability of agroforestry systems. Given their high vigour and productivity, their use may fit in with the ongoing development of new agroforestry systems in the region, whilst preserving cup quality as our results indicate. On the other hand, irrational use in a full sunlight system would increase ecological problems, since higher yields would have to be compensated for by additional fertiliser applications.

The recent development of high capacity methods for analysing the structure and function of genes, which may be collectively termed 'genomics', represents a new paradigm with broad implications. Although currently available for only a few model plants, it seems likely that such information will rapidly become available for most widely studied plant species such as *C. arabica*. The advent of large-scale molecular genomics will provide an access to previously inaccessible sources of genetic variation, which could be exploited in breeding programmes. Anticipated outcomes in coffee breeding include (i) rapid characterisation and managing of germplasm resources, (ii) enhanced understanding of the genetic control of priority traits, (iii) identification of candidate genes or tightly linked genomic regions underlying important traits, and (iv) identification of accessions in genetic collections with variants of genomic regions or alleles of candidate genes having a favourable impact on priority traits. In this way, the recent efforts to set up an international commitment (ICGN) to work jointly for the development of common sets of genomic tools, plant populations and concepts would be extremely useful.

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Chapter 15
Tea Breeding

Tapan K. Mondal

15.1 Introduction

People drink three non-alcoholic beverages from nature's resources, namely, tea, coffee and cocoa. Among these, tea is the most important drink for two-thirds of the world population due to its attractive aroma, taste and health benefits. It is a safe and easily affordable drink for all sections of the society. It has evoked great interest in the medical community in the last few decades as it shows with scientific evidence to prevent a number of human ailments.

Tea (*Camellia sinensis* L.) of family Theaceae is a woody, perennial plantation crop (Fig 15.1a,b) with an economic life span of more than 60 years. Tea has become one of the most important agro-based, eco-friendly, labour intensive, employment generating, export-oriented industries in all the tea-growing countries. Tea occupies 2.7 million hectares of land all over the world with an annual global production of about 3.0 million tones (Table 15.1). Despite occupying only 16.4% of the total tea growing area of the world, India ranks first as a producer, consumer and exporter of black tea. However, because of its ever-increasing domestic consumption, India is still incapable of exporting more than 15.4% of the total production to the world tea market. Commercially, it contributes around 5.27% share on Indian gross national product and provides direct employment to about one million and indirect employment to about two million people. While India registered 835,200 tons of tea in 2004, China being the second largest producer accounts 820,000 tons in 2004 (Yajun et al. 2005).

15.1.1 Historical Aspects

Tea plants are believed to have been discovered accidentally by the Chinese legendary Buddhist Emperor Sheng Nang around 2737 B.C. So it is assumed

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Fig. 15.1 (**A**) A mature seed bearing tea plant. (**B**) A view of tea plantation where harvesting is in progress

that tea was being used as a drink for nearly 5,000 years. As soon as the medicinal value began to be attributed to tea by the Chinese, a demand for supplies sprang up that resulted in the cultivation of tea in Sichuan province about 3,000 years ago.

Subsequently, the knowledge of tea cultivation spread everywhere by the Buddhist pilgrimage with fine arts of Buddhism. Although in India wild tea plant was discovered by Major Robert Bruce in Assam in 1823, seeds were also brought by G.J. Gordon from China during 1834 for establishing a commercial garden. Later, C.A. Bruce, superintendent of the governmental tea plantation, took active interest

Table 15.1 Tea production in major producing countries of the world (million kg)*

Country	1951	1991	1995	1996	1997	1998	1999	2000	2001	2002	2003
Argentina	0	44.0	32.0	43.0	55.0	50.0	50.0	63.0	59.0	58.0	58.0
Bangladesh	24.0	45.0	48.0	53.0	53.0	56.0	47.0	52.6	57.3	52.9	56.8
China	65.0	542.0	588.0	593.0	613.0	665.0	655.0	683.0	702.0	745.4	770.0
India	285.0	754.0	756.0	780.0	811.0	870.0	805.0	846.5	854.0	826.0	857.0
Indonesia	48.0	153.0	144.0	164.0	154.0	166.0	154.0	157.0	173.0	173.0	168.0
Japan	44.0	88.0	85.0	89.0	91.0	83.0	88.5	89.3	90.0	84.0	87.0
Kenya	7.0	204.0	245.0	257.0	221.0	294.0	248.0	236.0	295.0	287.0	294.0
Sri Lanka	148.0	242.0	246.0	259.0	277.0	280.0	283.0	307.0	296.0	310.6	303.3
Vietnam	2.0	33.0	40.0	40.0	52.2.0	56.6	65.0	70.0	80.0	84.0	78.0
Others	30.0	315.0	272.0	295.0	331.0	379.0	39	29.3	33.3	33.8	36.5
Total	653	2581	2521	2639	2724	2963	2830	2908	3041	3056	3097

*Different volumes of Tea Statistics, Tea Board, India.

in the cultivation of indigenous tea plant. Soon commercial interests moved in and the world's first privately owned Tea Company – the Assam Tea Company – was established at Jorhat, Assam on 12 February 1839 with the directives from British Parliament. This was the beginning of the present day Tea Industry in India.

15.1.2 Origin and Distribution

Southeast Asia is the original home for tea. According to Wight (1959), the primary centre of origin of tea is considered to be around the point of intersection of latitude 29°N and longitude 98°E near the source of the river of Irrawaddy, the point of confluence where lands of Assam, North Myanmar, southwest China and Tibet meet. Secondary centres of origin are considered to be located in southeast China, Mizoram and Meghalaya (Kingdon-Ward 1950). The above areas are, therefore, considered to be the zone of origin and dispersion of the genus *Camellia* as a whole (Sealy 1958). However, presently tea cultivation is spread within the latitudinal range of 45°N–34°S.

Tea was introduced to Japan from China in the early part of the eighth century. From Japan, tea cultivation extended to Indonesia during the seventeenth century. In Sri Lanka, tea was first planted in 1839 when seeds were brought from India (Calcutta). In USSR, tea cultivation started when seeds were imported from China towards the end of last century. Later, from USSR, seeds were exported to Turkey during 1939–1940. In Europe, tea was introduced in 1740 by the East India Company's Captain Goff, but those planted in the Royal Botanic Garden at Kew in England could not survive (Sealy 1958). The first successful introduction was achieved by a British merchant and naturalist John Ellis in 1768 (Aiton 1789; Booth 1830). From there tea cultivation spread to the African countries at the end of the 19th century. Presently, more than 52 countries produce tea.

15.1.3 Botanical Aspects

A summary of morphological characters on the three types of tea are given below (Barua 1963):

China type (*C. sinensis*): This is a shrub (1–3 m tall) with many stems arising from the base. The relatively small, thick and leathery leaves have stomata that appear to be sunken in the lamina. Short and stout petioles give the leaf an erect pose and are usually 3–7 in number. The flowers that are borne singly or in pairs in the axils have 6–10 mm long pedicels with 2–3 sub-opposite scars. The flowers are characterised by 7–8 cup-shaped, 1.5–2.0 cm long and broad-oval to sub-orbicular petals with about 3–5 styles that are generally free for greater part of their length but occasionally free upto the base of the ovary. The capsules have 1, 2 or 3 locules containing 1–3 nearly spherical seeds of about 10–15 mm diameter.

Assam type (*C. assamica*, Masters): It is a 10–15 m high tree with a trunk and robust branching system. The relatively large, thin, glossy leaves with more or less acuminate apex have distinct marginal veins and broadly elliptic leaf blades that are usually 8–20 cm long and 3.5–7.5 cm wide. Single or paired pedicellate flowers have smooth and green scars of 3 bracteoles, numerous stamens, 5–6 persistent sepals with 7–8 white petals.

Cambod type (*C. assamica* sub sp. *lasiocalyx*. (Planch. ex Watt.): This is an upright tree (6–10 m tall), with several, almost equally developed branches and more or less erect, glossy, light green to coppery-yellow or pinkish red leaves, the size of which is intermediate between *C. sinensis* and *C. assamica*. Although the flowers are more or less similar to the Assam type yet, they have 4 or more bracteoles, 3–4 ovules with 5 locules and 3–5 styles that are free up to half the length.

15.1.4 Genome Size and Diversity

The genome size in terms of 4C DNA amount for *Camellia sinensis* is 15.61 ± 1.06 where 1C DNA is equal to 3824 mega base pair (Mbp) and 1 pg = 980 Mbp (Hanson et al. 2001). Generally, tea chromosomes are small and tend to clump together due to 'stickiness'. Tea is diploid ($2n = 30$; basic chromosome number, $x = 15$) and karyotype ranges from 1.28 μm to 3.44 μm (Bezbaruah 1971). The r value (ratio of long arm to short arm) for all the 15 pairs of chromosomes ranges from 1.00 to 1.91. This consistency in diploid chromosome number suggests a monophyletic origin for all *Camellia* species. However, few higher ploidy level, such as triploids, for example, TV-29, HS-10 A, UPASI-3, UPASI-20 ($2n = 45$), tetraploids ($2n = 60$), pentaploids ($2n = 75$) and aneuploids ($2n \pm 1$ to 29) have also been identified (Singh 1980; Zhan et al. 1987).

Owing to extensive internal hybridisation between different *Camellia* taxa, several intergrades, introgresants and putative hybrids have been formed. These can be arranged in a line based on morphological characters that extend from China types through intermediates to those of Assam types. Indeed, because of the extreme homogenisation, the existence of the archetypes of tea is doubtful (Visser 1969). Till date, the numerous hybrids currently available are still referred to as China, Assam or Cambod tea depending on their morphological proximity to the main taxa (Banerjee 1992). Tea breeds well with wild relatives and thus tea taxonomists have always been interested to identify such hybrids due to their suspected involvement in the genetic pool of tea. Two particularly interesting taxa are *C. irrawadiensis* and *C. taliensis* whose morphological distribution overlaps with that of tea (Banerjee 1992). It has also been postulated that some desirable traits, such as anthocyanin pigmentation or special quality characters of Darjeeling tea might have been introduced to tea gene pool from wild species (Wood and Barua 1958). Other *Camellia* species, which are suspected to have contributed to the tea genetic pool by hybridisation include *C. flava* (Pifard) Sealy, *C. petelotii* (Merrill) Sealy (Wight 1962) and possibly *C. lutescens* Dyer (Sharma and Venkataramani 1974). The role of *C. taliensis* is,

however, not clear because the species itself is considered to be a hybrid between *C. sinensis* and *C. irrawadiensis* (Wood and Barua 1958; Visser 1969). Therefore, it is generally agreed that at least three taxa, that is, *C. assamica*; C. *sinensis; C. assamica* sub sp. *lasiocalyx* and to an extent *C. irrawadiensis* have mainly contributed to the genetic pool of tea. The term 'tea' should therefore cover progenies of these taxa and the hybrids thereof or between them.

Apart from this natural diversity, the different tea research institutes and dedicated planters have further developed a number of cultivated varieties with better yield, quality and traits, such as resistance to drought, diseases, and so forth. In our estimation, more than 600 such cultivars have been released for commercial cultivation worldwide and many of them have special characters (Table 15.2).

Table 15.2 Different tea cultivars with special characters

No	Special characters	Clone	Originator	Reference
1.	Wind tolerance	UPASI-2, UPASI-10	UPASI-TRF, India	Sharma and Satyanarayana (1987)
2.	Drought resistance	UPASI-9	UPASI-TRF, India	Sharma and Satyanarayana (1987)
3.	Frost resistance/tolerance	BS 53	HPKV-TES, India	Deka et al. (2005)
4.	Small leaf	CH-1	IHBT, India	Mr Shekhawat (Personal communication)
5.	Biggest leaf	Betjan	Betjan T.E, India	Singh (1980)
6.	Blister blight tolerance	TRI-2043, DT-1	TRI, Sri Lanka	Sivapalan et al. (1995)
7.	High pubescence content	TRI/2043	TRI, Sri Lanka	Sivapalan et al. (1995)
8.	High anthocyanin pigmentation	TRI/2025	TRI, Sri Lanka	Sivapalan et al. (1995)
9.	High tolerance to pH	TN-14-3	TRF, Kenya	Anonymous (1999)
10.	Poor fermenter	12/2	TRF, Kenya	Anonymous (1999)
11.	Mite tolerance	7/9	TRF, Kenya	Anonymous (1999)
12.	Scale insect tolerant	TN 14-3	TRF, Kenya	Anonymous (1999)
13.	High polyphenol content (53.7%)	Luxi white tea	TRI, China	Yu and Xu (1999)
14.	High amino acid content (6.5%)	Anji white tea	TRI, China	Yu and Xu (1999)
15.	Low caffeine content (0.14%),	Guangdong tea	TRI, China	Yu and Xu (1999)
16.	High caffeine content (6.96%)	Wild tea at Yunnan	TRI, China	Yu and Xu (1999)
17.	Water logged tolerant	TV-9	TES, Assam, India	Singh (1980)

UPASI-TRF=United Planter's Association of South India-Tea Research Foundation, Tamil Nadu, India; HPKV=Himachal Pradesh Krishi Visawavidalaya, Himachal Pradesh, India; IHBT=Institute of Himalayan Bioresource Technology, Himachal Pradesh, India; TRF=Tea Research Foundation; TRI=Tea Research Institute; TES=Tocklai Experimental Station, Jorhat, India

15.1.5 Economic Importance

The economic importance of the genus *Camellia* is primarily due to tea. Apart from being used as a beverage, leaves of tea are also used as vegetables like the 'leppet tea' in Burma and 'meing tea' in Thailand. While *C. oleracea* produces oil, used in the cosmetic industry, *C sasanqua, C. japonica* and *C reticulata* have great importance due to their ornamental values. Tea seeds yield about 17.3% of oil compared to *C. sasanqua* (58%) and *C. japonica* (66%). The oil is of non-drying class and resembles that of *C. sasanqua*. Although the oil is used as a lubricant, extraction of oil from tea seed is not economically viable (Wealth of India 1950). Moreover, tea seed cakes also contain saponins. Although it has a poor value as a fertiliser and is unfit for animal feed on account of its low nitrogen, phosphorus and potassium contents, yet it is being successfully utilised in the manufacture of a nematocide (Wealth of India 1950).

Though tea is mainly consumed in the form of 'fermented tea' or 'black tea', yet 'non-fermented' or 'green tea' and lesser known 'semi-fermented' or 'oolong tea' are also available. These types vary in their method of manufacture, chemical constituent, appearance and organoleptic taste. While black tea is widely used in India and other European countries, green tea is popular in China, Japan, Indonesia and Taiwan. Oolong tea is mainly consumed in some parts of China as well as Taiwan. Worldwide 80% black tea, 18% green tea and 2% oolong tea are being produced.

For black tea, the young tender leaves are completely fermented after withering. The fermentation results in oxidation and polymerisation of polyphenols, changing the nature of the chemicals constituents of tea leaves and forming theaflavin and thearubigin. These polyphenols are responsible for the briskness, strength, colour, taste, aroma and pungency associated with black tea. The infusion of black tea has a bright red or copper colour, astringent taste and characteristic aroma. On the other hand, green tea is unfermented and is the least processed among the three types. The plucked leaves are harvested and steamed immediately to inactivate the enzymes to prevent oxidation and polymerisation of primary polyphenols, which results in retaining of green colour in the finish product. Green tea infusion has a leafy taste with a smell of fresh vegetables and low caffeine content. In oolong tea, primary polyphenols are allowed to partly oxidise.

Oolong tea is not common and is intermediate in characteristic between green and black tea. Immediately after plucking, the tea leaves are partially fermented for about half the time of black tea. It has the colour of black tea and flavour of green tea.

Tea was used initially as medicine, later as beverage and has a proven future potential of becoming an important industrial and pharmaceutical raw material. Scientific reports in the last two decades have validated many beneficial claims of tea. Majority of the beneficial effects have been attributed to the polyphenolic constituents. Several studies suggest that phenolics may be of importance in reducing the incidence of degenerative diseases, such as cancer and arteriosclerosis. The most relevant compounds in dietary regime are cinamic acid derivatives and flavonoids.

As natural polyphenols remain unchanged in green tea, it can be said that green tea is more beneficial than black tea. The strong anti-oxidant potential of tea polyphenol (Wiseman et al. 1977; Allemain 1999) is thought to be responsible for most of the beneficial effects of tea. Tea catechins have been found to be better antioxidants than vitamin C, E, tocopherol and carotene. The anti-oxidants activity of tea polyphenols is not only due to their ability to scavenge superoxides, but also due to the increase in activity of antioxidant enzymes, for example, glutathione peroxidase, glutathione reductase and glutathione-*S*-transferase. However, the anti-oxidant activity of tea is diminished by the addition of milk to the infusion due to binding of tea polyphenols to milk proteins. Tea polyphenols and flavonoids have been reported to show the cardiovascular activity by inhibiting either enzymatic or non-enzymatic lipid peroxidation.Tea polyphenols also inhibit the absorption of dietary fats and cholesterols (Chen et al. 2000). Several experimental evidences point to the potential of tea to protect against cancer at several stages of carcinogenesis including cancer prevention, endogenous carcinogenic activation, DNA damage and destabilisation, cell proliferation, neoplastic growth and metastasis. Tea, especially green tea reduces the incidence of cancers of the stomach, small intestine, pancreas, lung, breast, skin, urinary bladder, prostrate, oesophagus and mouth (Vasisht et al. 2003). Green tea drinking also has been shown to possess anti-diabetic activity (Gomes et al. 1995), anti-arthritic activity (Tapiero et al. 2002), anti-plaque activity (Yu et al. 1995), anti-viral activity (Okubo and Juneja 1997), anti-AID activity (Hashimoto et al. 1996), anorectic effect (Kwanashie et al. 1989) and anti-microbial activity (Hamilton-Miller 1995). It has also been reported recently that green tea polyphenols exhibit neuromuscular, anti-angiogenic, anti-hepatotoxic, anti-proliferative/apoptotic and immunomodulatory effects (Sueoka et al. 2001).

15.2 Conventional Breeding

15.2.1 Methods of Propagation

Seeds: Conventionally tea is propagated either through seeds or cuttings. Seeds are generally produced in ‘seed bari’ (seed orchard). A fully matured healthy seed while attaching to the plants or immediately shaded are collected from the ground of the seed orchard. This is primarily due to the fact that tea seeds being recalcitrant have low viability. To differentiate the good with hollow, seeds are subjected to sinker floater test where seeds are soaked over night in water. After that only sinkers are selected and planted in sand bed with scar mark down for stratification. After 45 days, the germinated seeds are transferred to the polythene sleeve and kept under the shaded nursery for another 12–18 months.

Cuttings: Seeds were the only commercial method for propagation till the beginning of nineteenth century. However, due to out-breeding nature of the plant, seedling shows a wide variability for the attributes, such as yield, quality, and so forth. Simultaneously, the need for rapid multiplication of some merit tea plants

forced people to find some alternatives. The first attempt for vegetative propagation of tea was done in Indonesia by budding as well as grafting. However, due to slower speed, this method could not serve the purpose of rapid multiplication. Hence, the faster propagation by single leaf cutting was developed simultaneously in India, Sri Lanka and Indonesia (Keuchenius 1923). This was further fine tuned later to fit for commercial venture that exists now.

Cuttings from green and semi-hard wood are usually taken from current-year growth. For single node cuttings, the cut should be slanted, as close as possible to the axillary bud. Cuttings are then immediately subjected to fungicide as well as commercial grade rooting-hormone treatment and inserted in the nursery for root induction for 45–60 days depending on the location, planting material and so forth. The successful rooted cuttings are then transferred to polythene sleeves filled with a good virgin soil (pH around 4.5) having adequate water holding capacity and kept for another 8–12 month in the nursery, which by then become ready for filed transfer (Fig. 15.2).

Grafting: In recent years, grafting as an alternative propagation technique has gained considerable popularity. In this technique, fresh single leaf internode cuttings of both rootstock and scion are generally taken (Fig. 15.3). Scion commonly a quality cultivar is grafted on rootstock, which is either drought tolerant or high yielding cultivar. On grafting, the scion and stock influence each other and thus composite plants combine both the characters, resulting in 100% increase of yield with better quality than either of the ungrafted cultivar. Recently, a modified improved 'second generation' grafting has been developed, where a tender shoot was grafted on the young seedlings of tea, which has an additional advantage over conventional grafting due to presence of tap root system (Prakash et al. 1999). Nevertheless, with the increasing demand for clonal tea vegetative propagation with single leaf internode cuttings remains the best choice in the tea industry worldwide.

Fig. 15.2 Around 18-month-old tea plants ready to be transfer to the field

Fig. 15.3 Photograph of young seedling tea grafted plant (Source: Prakash et al. 1999)

15.2.2 Floral and Seed Biology

The first description on the significant difference in flower description between the China and Assam type of tea was made by Wu (1964), though there are several reports (Cohen Stuart 1916; Bakhtadze 1931; Wellensiek 1938; Barua 1970; Bezbaruah 1975; Rogers 1975) from all the tea growing regions. Characters, such as length of the style and style arm, number and length of the outer stamens or the size of the inner petals were able to indicate the difference in floral characters among the varieties. Importantly, anatomical study suggested that tea flower should be classified as central placental type instead of parental placental type (Wu 1964).

Tea plants showed an appreciable degree of self sterility and invariably set a better crop of seeds with pollen from another bush nearly four times than that of selfed seeds (Wight 1938; Wu 1964). Generally selfed seeds exhibited reduced germination. Progenies of self-pollination were inferior in vigour to those of cross-pollination. Investigation on the mechanism of pollination revealed that tea pollens are heavy and sticky in nature and occur mostly in clumps, a condition, which is not favourable for carrying out by wind, rather only non-viable dried up pollen grains can move long distance by wind. However, insects, such as bees and wasps (*Hymenoptera*) carry pollen from bush to bush. Besides, Bezbaruah (1975) observed that syrphid flies (*Diptera*) are the most common insects for natural cross-pollination. The tea flower secretes considerable amount of honey but they contain high phenols that causes indigestion. Perhaps this may be the reason for not

attracting the insects for pollination (Barua 1989). Therefore, for production of high seed set, it may be necessary to take measures to attract insects in tea seed orchards.

After 24–48 h of pollination, the corolla withers-off and drops from the pedicel along with the anther lobes leaving the ovary exposed. The persistent calyx lobes close flatly over the ovary and the style as well and the stigma gradually withers off. Though, pollination takes place during flowering growth, that is September–January in India, the first external sign of development of fruits become evident by March and gain considerable size by May. By about August, the fruits attain its full size with completely developed embryo and cotyledon. The mature embryo with two large cotyledons remains covered by a hard, deep brown testa, formed by the outer integument. The pericarp encloses 1–3 seeds inside and is made of thick, parenchymatous tissue when young but becomes sclerotic on maturity. The ripening of fruit generally takes 12 months from the time of flowering till maturity (Singh 1999).

Generally 1–3 seed of 1.5–2.0 cm diameter are found in each capsule. The seeds have a hard testa outside and the embryo is covered in between two large cotyledons. Tea seeds are recalcitrant, that is, they lose viability within a few days after shedding from the plant. (Bhattacharjee and Singh 1994). However, their viability can be maintained by surface sterilising with 0.01% mercuric chloride solution for 15 minutes and subsequent cold storage at 4°C. Though, seeds are generally stored in moist charcoal for some days, yet it is advisable to use the healthy seeds for propagation as early as possible (Singh 1999).

15.2.3 Breeding Objectives

Tea breeding program varies from country to country, depending on the local needs, which is illustrated below (Table 15.3). However, by and large it is aimed at for improving quality and yield.

However, generally the breeding work at black tea producing countries, such as India, Kenya and Sri Lanka are biased towards the developing of high yielding and quality clones, whereas the tea producing countries near equator, such as Japan, China, and so on, are focused on the development of cold resistant, frost resistant, and so forth, as these countries also primarily produced green tea where quality of made tea does not have much influence on price.

15.2.4 Breeding Techniques

15.2.4.1 Hybridisation

In natural hybridisation, based on known characters, such as previous performance of yield, quality or diseases resistancy, two parents are planted side by side in an isolated place and allowed to bear fruits. Subsequently the seeds (F_1) are harvested, raised and planted. If average performances of these plants are found to be better than either parent, then seeds are released as hybrid or bi-clonal seed. However,

Table 15.3 Breeding objectives of tea

Objectives	Importance	Region
Improving quality	Directly linked to the profitability	Black tea producing countries such as India, East-Africa, Sri Lanka, Bangladesh and Indonesia
Increasing yield	Horizontal increase of production by extension planting is limited worldwide	Worldwide
Drought tolerance	Reduce productivity and occur all tea-growing regions of the world.	Worldwide where tea grown as rain-fed crop
Reduce winter dormancy	No leaf production during winter months and occurs in North-East India, Japan and China, etc.	Tea plantation in near equator
Hail/Frost resistance	Causes economic loss as young leaves during rainy season are mostly affected. Generally occurs in the hilly region.	Hilly region of the tea producing countries
Water log tolerance	Reduce productivity during rainy season. Generally occurs in North-Eastern India	North-East India
Cold hardiness	Reduced productivity during winter due to snow. Generally occurs in China, Japan and Russia	Mainly in Japan, Russia and China
Diseases resistance, such as blister blight, stem canker, etc.	Blister blight causes severe damage as only young leaves are infected. Generally occurs in Japan, Sri Lanka, South India and Darjeeling hills of North-East India.	Mainly India, Sri Lanka, Indonesia and Japan
Pest resistance. such as red spider mite, tea mosquito bug, leaf sucking pest, etc	Most important biotic stress as all causes severe damage to the leaves. Generally, occurs in all the tea-growing regions in the world.	Worldwide
Suitability to type of manufacturing	For matching the customer's demand as well as better recovery percentage in made tea	Black tea producing countries such as India, East-Africa, Sri Lanka, Bangladesh and Indonesia
Low input responsive clone	Required for organic tea farming	Organic tea

some of the outstanding performers among these progenies are marked and verified for multi-location trial and still, if found suitable released as clone. These clones are geographically specific and most of the tea research institute of the world has generated the clone for their own region. Some time in the above process more than two parents are used and known as polyclonal seeds. The idea is to introduce more

variability among the F_1 seeds. Since it is difficult to know about the pedigree of the cultivar (as pollen may come from any male), hence the chance of reproducibility is low, and therefore the process thus least preferred currently.

Hand pollination or control cross, despite being an important approach has made a limited success in tea breeding. The reasons identified could be (i) low success rate, (ii) availability (2–3 months) of tea flower for a short period. (3) longer time taken for seed maturation (12–18 months) (4) difference in flowering time for different clones. Few clones have been released in Kenya and Malawi recently using this procedure.

15.2.4.2 Selection

The seeds from a particular seed bari (garden) were known by the name of that garden or locality and was called a *Jat* and those *jats* were the main source of planting materials of tea. There were wide variations in morphology, yield and quality of tea produced from a particular plot even though the source of seed was the same due to high out breeding in nature. Wight (1939) showed that about 10% of the bushes in a commercial tea garden in North-East India produced only 2% of the total crops and about 0.5% bushes produced as much as or more than 300% of the average yield. From the very beginning, the planters thought that new areas planted with seeds from more uniform plots produced better quality tea and those selected plants were used for future plantations. Thus, the process of selection started. The first scientific attempt to select improved tea in North-East India was made by Stiefelhagen brothers in 1860 by establishing standard sources of tea seeds. Subsequently, scientific improvement of tea by selection was followed in many countries, such as Indonesia (Wellensiek 1934), Java (Cohen Stuart 1929), Russia (Bakhtadze 1935) and in North-East India (Wight 1939). Mother bushes were selected based on morphological characters followed by anatomical (Wight 1956) as well as chemical constituents (Timoshenko 1936). Indigenous Assam tea was improved by following the technique of mass selection. The yield increased considerably, because of line breeding for desirable morphological features that are genetically linked with the characteristics of Assam tea. After that, line breeding techniques were followed to improve further by mainly evolving more uniform tea plant with better quality and adaptability. In the earliest effort, two selected *jats* were hybridised to combine desirable characters into their progenies. The 'Rajghur' *jat* was developed by combining high quality of a light leaf local jat with the vigour of a dark leaf 'Manipuri' jat.

However, the seed grown plants were not uniform as their characters were governed by genotypes of their parents, which were diverse in some phenotypic characters in relation to environmental and soil conditions. In some cases, the yield and quality were unpredictable. It was, therefore, felt necessary to develop clonal cultivars in tea like many other fruit crops by multiplying the selected bushes vegetatively. Meanwhile, propagation by cuttings was attempted in several parts of the tea growing areas around the world (Tunstall 1931a,b; Tubbs 1932; Wellensiek 1933), however, standardisation of the technique of single leaf internode cuttings, practiced

today, took a long time to be successful. Following this technique, Tocklai Experimental Station released the first lot of three clones in 1949, which revolutionised the tea industry in North-East India and more clones have since been released from time to time.

Today all tea growing countries of the world have developed clonal materials as per their requirements. So far, the natural variability has been exploited to a limited extent to evolve better planting material as clones. The procedure of clonal selection in tea is shown in Fig. 15.4.

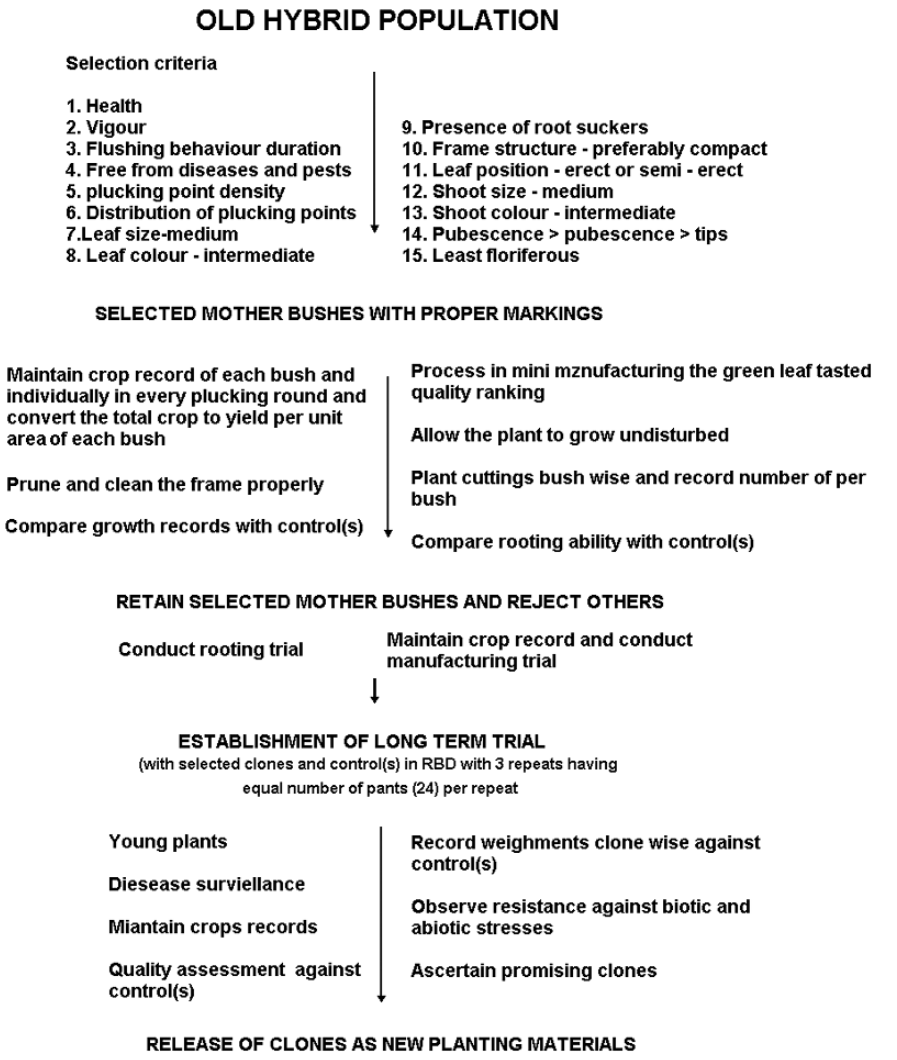
OLD HYBRID POPULATION
Selection criteria
1. Health
2. Vigour
3. Flushing behaviour duration
4. Free from diseases and pests
5. plucking point density
6. Distribution of plucking points
7.Leaf size-medium
8. Leaf colour - intermediate
9. Presence of root suckers
10. Frame structure - preferably compact
11. Leaf position - erect or semi - erect
12. Shoot size - medium
13. Shoot colour - intermediate
14. Pubescence > pubescence > tips
15. Least floriferous
SELECTED MOTHER BUSHES WITH PROPER MARKINGS
Maintain crop record of each bush and individually in every plucking round and convert the total crop to yield per unit area of each bush
Prune and clean the frame properly
Compare growth records with control(s)
Process in mini mznufacturing the green leaf tasted quality ranking
Allow the plant to grow undisturbed
Plant cuttings bush wise and record number of per bush
Compare rooting ability with control(s)
RETAIN SELECTED MOTHER BUSHES AND REJECT OTHERS
Conduct rooting trial
Maintain crop record and conduct manufacturing trial
ESTABLISHMENT OF LONG TERM TRIAL
(with selected clones and control(s) in RBD with 3 repeats having equal number of pants (24) per repeat
Young plants
Diesease surviellance
Miantain crops records
Quality assessment against control(s)
Record weighments clone wise against control(s)
Observe resistance against biotic and abiotic stresses
Ascertain promising clones
RELEASE OF CLONES AS NEW PLANTING MATERIALS

Fig. 15.4 Clonal selection scheme of tea (Singh 1999)

15.2.4.3 Polyploidy Breeding

Most of the tea plants under cultivation are diploid (2n = 30), although few natural triploids, tetraploids and pentaploids have also been reported (Karasawa 1932, 1935; Bezbaruah 1971, 1975; Wachira 1991). Triploids, in general, are more vigorous and hardier and tolerant to cold than diploids (Simura 1956a). Some tetraploids and aneuploids have also been found to possess superior vigour. Though, Bezbaruah (1971) reported that the quality of tetraploids and natural triploids are inferior to the diploids, yet, two commercial clones UPASI-3 and TV-29 are triploids, which produce acceptable quality of tea. Genetic variation or mutation of diploid tea plants into polyploid can, therefore, be expected to improve vigour and hardiness. Since tea polyploids are scarce under natural conditions, their artificial production has become a necessity. Breeding of polyploids of tea has been advocated by several workers (Bezbaruah 1975; Simura 1956a,b; Singh 1980). Attempts have also been made to create triploids artificially by hybridising tetraploids with diploids tea in Japan (Osone 1958), India (Choudhary 1979) and Bangladesh (Rashid et. al. 1985) but the success is low. At Tocklai Experimental Station, Assam over 170 and 70 polyploids through hybridisation and chemical treatments have been developed, respectively (Singh 1999). However, polyploid breeding is still in early stages of exploration, though possibilities are immense for production of better cultivars.

15.2.4.4 Mutation Breeding

The work on mutation breeding in India was initiated during 1967–1968 at Tocklai Experimental Station, Assam with the objectives of increasing genetic variability for possible use in evaluation of superior planting materials. However, except a preliminary report on irradiation with Υ rays on cuttings, no progress has been achieved till now (Singh 1984). Studies conducted elsewhere, (Tavadgiridze 1979) have shown that a wide range of variations can be created by irradiating various plant parts, like seeds, leaf cuttings, axillary and apical buds to induce mutation.

15.2.4.5 Interspecific Hybridisation

Tea breeds freely among the two species *sinensis* and *assamica*. However, the natural hybridisation between cultivated teas with other species is rare. Wight and Barua (1954) hybridised *C. irrawadiensis* with *C. sinensis*. Although the progenies were resembled later, they failed to attack tester tong due to inferior quality. Later Bezbaruah (1971) made a successful hybridisation between *C. Japonica* and *C. sinensis*. Though morphologically it was found to be intermediate but produced low yield as well as quality. However, a commercial clone TV-24 in Assam was produced from the cross between F_1 hybrids of *C. irrawadensis* as well as *C. assamica* and TV-2 an Assam-China hybrid.

15.2.5 Global Advances of Tea Breeding

Progress and achievements of tea breeding works in certain tea producing countries have been well reviewed (Ghosh-Hazra 2001; Singh 1999). From those literatures, it indicates the initial emphasis was to collect and evaluate either indigenous or exotic germplasm for befitting the local environment. However, with the increase of the region specific need of the industry, almost all tea producing countries have developed their clones or seed stocks, which are reviewed here.

The breeding work at India started at Tocklai Experimental Station, Tocklai, and since 1949 a total of 32 clones, namely, TV-1 (Tocklai Vegetative) to TV-32 have been developed. Among them, clone merits for quality is TV-21, yields are TV-29, TV-30 and TV-18, drought tolerant are TV-1, TV-17 and water logged tolerant is TV-9. Furthermore, 14 bioclonal seed stocks (Table 15.4) and 134 TRA/Garden series clones (Table 15.5) have been developed. A brief account of different tea clones are tabulated below.

In South India, the breeding work at UPASI, Tamil Nadu started during early 1960s, which has resulted in the release of 28 clones, namely, UPASI-1 (United Planter's Association of South India) to UPASI-27 and five bioclonal seed stocks. Among them, UPASI-9 is considered to be drought tolerant. Recently, they have also developed a clone UPASI TRF-1, which has highest yielding capacity with distinct morphological characters and quality attributes. Further to widen the genetic base, clones developed at Sri Lanka were introduced, experimented and found TRI-2024 and TRI-2025 were suitable. Development of biclonal seed stock was initiated in the late 1970s and early 1980s. Over the years, several cross combinations were tested and among them five bioclonal seed stocks, such as BSS-1 (UPASI-10 X TRI-2025), BSS-2 (UPASI-2 X TRI-2025), BSS-3 (UPASI-9 X TRI-2025), BSS-4 (UPASI-15 X TRI-2025), BSS-5 (CR6017 X UPASI-8) have been released by UPASI scientific department in 1993 (Sharma and Satyanarayana 1987).

Table 15.4 Description of bioclonal seedlings developed by Tocklai Experimental Station, (Singh 2004)

Cultivar	Parent combination	Year of release	Suitable for area
TS 378	14.5.35 X 14.6.28	1968	Hills (Darjeeling) Area
TS 379	14.5.35 X 14.12.16	1969	Hills (Darjeeling) Area
TS 397	TV-1 X 19.35.2	1976	Plains
TS 449	TV-1 X 270.2.14	1970	Plains
TS 450	TV-2 X 270.2.13	1970	Hills (Darjeeling)
TS 462	TV-1 X 124.48.8	1980	Plains
TS 463	TV-1 X TV-19	1984	Plains
TS 464	TV-1 X 19.29.2	1989	Plains
TS 491	TV-1 X S_3A_3	1989	Plains
TS 520	TV-19 X TV-20	1992	Plains
TS 506	TV-1 X 19.22.4	1994	Plains
TS 557	AV-2 X Teen Ali 17	1996	Hills (Darjeeling)
TS 569	AV-2 X Tukdah-78	1996	Hills (Darjeeling)
TS 589	TV-20 X Heeleakah 22/14	1996	Plains

Table 15.5 Different region specific garden series clones

Area	Clone
Darjeeling	Phoobsering 312, Phoobsering 1404, Phoobsering 1258, Kopati 1/1, Happy Valley 39, Bannockburn 157, Tukdah 145, AV2, Tukdah 253, Tukdah 246, Bannockburn 777, Rungli Rungliot 4/5, Bannockburn 688, Tukdah 78, Tukdah 383, Rungli Rungloit 17/144, CP-1, Teesta Valley 1, Badamtam 15/263, Balasun 7/1A/76, Balasun 9/3/76, Thurbo 3, Thurbo 9 and Lingia 12
South India	ATK-1 (drought tolerant clone), C-17, D-12/A2, C-1, CR-6017 (quality clone), SMP-1(resistant to blister blight), W-35, SA-6, TTL-1, TTL-2, TTl-4 and TTL-5
Kangra Valley	Kangra and Jawala
Tripura	Huplongcherra 18, Huplongcherra 26, Meghlibundh 11, Meghlibundh 20 and Meghlibundh 25
Barak Valley	Narinpore 4, Narinpore 18, Narinpore 22, Chandighat 9, Longai 17, Longai 26, Poloi 23 and Lalamookh 7
Dooars and Terai	Hantapara 12, Huldibari 19, Leesh River 9/34, Sukna 7, Sanyasithan 8, Kamalpur 6, Mohargung and Gulma 25

The plant improvement program at Sri Lanka started with procurement of some seeds of Betjan, Manipur and Rajghur from India (Assam), which were planted in Peradoniya Botanic Garden at Sri Lanka to establish the first tea garden during late 1930s. However, scientific works started only during 1937 when Dr F.R.Tubbs brought few seeds of ST 4/10 from Tocklai, Assam and seedlings were raised at Tea Research Institute and subsequently eight clones were developed (Richards 1966). Since then several clones were released, which are popularly known as 20 series, 30 series and 40 series clones. At present, Sri Lanka has more than 57% clonal tea area. Out of this, around 80% is composed of only three popular clones, that is TRI-2023, TRI-2025 and TRI-2026.

Bangladesh Tea Research Institute has developed 13 clones and 2 bioclonal seed stocks till now. These clones are known as BT-1 to BT-13. Among the two biclonal seed stocks, BST1 and BST-2, former are more popular, which is a cross between BT-1 and popular Indian tea cultivar, TV-1.

Initially, the Tea Research Institute of Vietnam has started the breeding work by collecting the planting material from India. Many good clones and seeds *Jats*, such as PH-1, PH-3 and IA have been evolved through selection and hybridisation (Tien 1993). Recently, two clones namely LDP1 and LDP 2 have been released with a yield potential of 17.5 ton green leaf/ha and 16.9 ton green leaf/ha, respectively. Two quality clones 276 and 215 (cross between PH1 and Shan tea) have also been developed, which will be released for commercial cultivation shortly (Tien 1993).

In Indonesia, the breeding works were initiated during 1980s and confined to selection only. Later through hybridisation, Tea and Cinchona Research Institute released ten clones, such as GMB-1 to GMB-10. While GMB-1 to GMB-5 has the potential to produce 3,500 kg/ha/year of made tea, GMB-6 to GMB-10 have

a productivity potential of 5,000 kg/ha/year. Few clones are also tolerant to blister blight (Arfin and Semangun 1999).

Tea improvement in Kenya started with the introduction of seeds from Assam, India during 1950s. Since these progenies had not been particularly selected for high yield and quality, the resultant seedling populations of mixed genotypes were genetically inferior, though diverse. However, they formed a good basis for improved seed jats and seedling through mass selection, which constituted second phase of mass selection. However, the limitations of phenotypic selection encouraged and spurred the search and development of superior genetically uniform tea clones from mid-1950s by Tea Research Institute of East-Africa, which has been continued later by Tea Research Foundation, Kenya. Presently 45 clones have been developed and out of which 24 were selected from seedling populations, including the most popular one, 6/8. Three other selections were bred from the combination of 6/8 and clone 31/11. Thus in total 27 clones shared the genetic pedigree of clone 6/8. It is noteworthy to mention that among the present cultivars, few clones merit some special characters, such as S 15/10, a high yielding clone recently developed and registered for 10,000 kg made tea/ha/annum under irrigated condition. Similarly SFS 150 and 303/577 account for drought resistant and TN 14-3 for resistant to high soil pH, SFS 150 and TN 14-3 for cold tolerant, 12/2 for poor fermenter, 311/287 for tetrapoid and 7/9, 57/15, SC 31/27, S 15/10 for tolerant to mite attack (Seurei 1996).

Presently there are more than 600 tea genotypes in China, of which 77 are for provincial clones, 76 are approved nationally and 20 are wild tea and rest is precious cultivars (Yongming 1999). However, among them only 54 are popular clones suitable for green tea, 32 cultivars for black tea and 33 cultivars are for oolong tea. Few cultivars such as Zhuyeqi and Fuding Dabaicha possess stronger prune-shock with higher plucking surface, more branches and shoots than that of popular clone ‘Xianggbolu’, thus the former two are recommended for mechanical plucking.

In Japan, the tea breeding was started way back in 1920 at Tea Experimental Station, Shizuoka. In addition, several private tea breeders started the varietal improvement of tea, which results in the development of many clones. Among them, Hikosaburo Sugiyama (1857–1941) a noted tea breeder popularly known as ‘Burbank of tea’ after U.S plant scientist Luther Burbank developed the most popular clone of Japan ‘Yabukita’, ‘Koyanishi’ and ‘Rokuro’. At present Yabukita alone is cultivated for more than 76% of all Japanese tea plantations. However, clonal selection program was more intensified during 1950 as a result, many good clonal cultivars were released in 1970. These newly developed cultivars have contributed much to the modern Japanese tea industry. Presently there are 70 registered varieties and few of them are Ooiwase, Yabukita, Surugawase, Sayamakaori, Yamakai, Kurasawa and Kanayamidori, Okuhikari and Sawamizuka (Takeo 1992).

15.2.6 Impediments for Tea Breeding

Although, conventional tea breeding is well established and contributed much for tea improvement over the past several decades, but time consuming and labour

intensive. The bottlenecks of conventional breeding are (1) perennial nature, (2) long gestation periods, (3) high inbreeding depression, (4) self-incompatibility, (5) unavailability of distinct mutant of different biotic and abiotic stress, (6) lack of distinct selection criteria, (7) low success rate of hand pollination, (8) short flowering time (2–3 months), (9) long duration for seed maturation (12–18 months), (10) clonal difference of flowering time and fruit bearing capability of some clones. Similarly, vegetative propagation is an effective method of tea propagation, yet it is limited by several factors, such as: (1) slower rates of propagation, (2) unavailability of suitable planting material due to winter dormancy, drought in some tea growing areas and so on, (3) poor survival rate at nursery due to poor root formation of some clones and (4) seasonal dependent rooting ability of the cuttings. Therefore, to overcome the problems related to tea breeding, scientists across the world, started finding some alternatives through biotechnological approaches which are discussed subsequently.

15.3 In Vitro Breeding

15.3.1 Micro-propagation

Several reviews on micro-propagation of tea and related species have been published in the past (Vieitez, et al. 1992; Dood 1994; Das 2001; Mondal et al. 2004). It is evident from the available literature that while Forrest (1969) was a pioneer for initiating the work on tissue culture of tea, Kato (1982) did a systematic study on micro-propagation of tea. Since then till late 1980s thrust of tea micro-propagation was on increasing the multiplication rate. Several factors which were found to influence the micro-propagation are explants (Kato 1996), basal medium either full or half-strength MS salts (Murashige and Skoog 1962), plant growth regulators (PGRs), such as 6-benzyladenine (BA), indole-3-butyric acid (IBA), thidiazuron (TDZ) for tea (Phukan and Mitra 1984; Banerjee and Agarwal 1990; Agarwal et al. 1992; Mondal et al. 1998). Further, manipulation of vitamin compositions along with organic and inorganic salts of MS, were found suitable for initiation and multiplication of axillary shoots of tea (Arulpragasam and Latiff 1986). The significant role of growth adjuvant for micro-propagation of tea is well documented. These include coconut milk (Agarwal et al. 1992; Rajkumar and Ayyappan 1992), yeast extract (Phukan and Mitra 1984; Sarwar 1985; Banerjee and Agarwal 1990; Phukan and Mitra 1990), casein acid hydrolysate (Chen and Liao 1983; Jha and Sen 1992), serine and glutamine as nitrogen sources (Chen and Liao 1982), and so on. Among the different carbon source, sucrose with the concentration between 15–60 g/l remains unanimous choice for tea micro-propagation (Nakamura 1990). However, the emphasis on survival rate including conventional (Sharma et al. 1999), biological hardening (Pandey et al. 2000) and micrografting (Prakash et al. 1999; Mondal et al. 2005) to increase survival rates of micropropagated tea was given only during early 1990s.

Studies on field performance of micropropagated tea and commercial exploitation started only at the beginning of the new millennium (Sharma et al. 1999; Mondal et al. 2004).

15.3.2 Somatic Embryogenesis

Somatic embryogenesis is considered to be the most efficient regeneration system of tea. Several reviews have been documented in recent past (Akula and Akula 1999; Mondal et al. 2004) which indicates that though Wu et al. (1981) were pioneers for somatic embryogenesis in tea, the technology has been applied for several purposes today, such as clonal propagation (Mondal et al. 2001a), genetic transformation (Mondal et al. 2001c), artificial seed production (Mondal et al. 2000b), some inter-specific hybrid crosses of *Camellia* (Nadamitsu et al. 1986) and androgenic or haploid plant production of tea (Chen and Liao 1982). Somatic embryogenesis of tea depends upon several factors, such as explants type (Akula and Akula 1999), time of cotyledon culture (Mondal et al. 2001a) and genotypes (Kato 1996).

Type, concentration and time of application of different PGRs have also been extensively worked out. In general, a high cytokinin-to-low auxin ratio or low cytokinin alone was found to be necessary for the induction of somatic embryos in tea, even reduction or omission of cytokinin in subsequent sub-culturing is also known. Generally, BA (0–10 mg/l) has been widely used for tea and related species (Zhuang and Liang 1985) followed by kinetin (0.05–10 mg/l) (Wachira and Ogado 1995). Among the different auxins, IBA (0–2 mg/l) was used mostly for somatic embryo induction in tea. However, different concentrations of NAA (Balasubramanian et al. 2000), 2,4-D (Bano et al. 1991), IAA (Sood et al. 1993) and α-napthoxyacetic acid (NOA), tetraphenylboron (TPB), phenylboronic acid (PBOA) (Ponsamuel et al. 1996) were also used.

Apart from growth regulators, some other factors, such as nitrate salts of potassium and ammonium, together with sulphate salts of aluminum, potassium, magnesium and ammonium, sucrose concentration as well as maltose and trans-cinamic acid (t-CA) (Mondal 2002c), Brassin, a synthetic analogue of a naturally occurring brassionoide (Ponsamuel et al. 1996) also has positive role for tea embryogenesis.

Hitherto, emphasis has been given to manipulate the nutrient composition, growth regulators in culture medium, physical conditions of incubation and other stress treatments to induce somatic embryos. However, induction of in vivo embryogenesis of tea could be achieved without using the conventional tissue culture media (Mondal et al. 2001d). Although the reason for this observation is not clear currently, seeds of *Camellia* appear to have a considerable inherent capacity for embryogenesis. Thus, at the right physiological stage, with appropriate levels of internal hormone and appropriate moisture profile of the substrate under sterile conditions, the tea seeds are able to produce embryos without any exogenous nutrient.

Information on hardening of tea somatic embryo derived plants is scant in the literature. Although Wu et al. (1981) were the first to transfer the plantlets to soil,

the composition of soil mixture and other conditions used by them, was not mentioned clearly. Later, Kato (1996) grew healthy tea somatic seedlings under natural conditions by transferring them into a mixture of vermiculite and soil (1:1) and they were covered under the plastic. On the other hand, Jha et al. (1992) hardened tea plantlets for 8 weeks in quarter-strength MS salts before transplanting to pots containing a mixture of peat and soil (1:1) and achieved an acclimatisation rate of 70%. Wachira and Ogado (1995) reported that multiple shoots differentiated from the germinated embryos were successfully rooted in mixture of sand: peat (3:1) in small pots. In order to improve rooting, Ponsamuel et al. (1996) treated the plantlets with 1mM indole-3-acetonitrile, 1 mM brassin and 10 mM phloroglucinol in liquid MS medium for 15 days and after profuse root proliferation, the plantlets were acclimatised in pots containing vermiculite. Eventually the plants were transplanted in the greenhouse.

Akula and Akula (1999), transferred the small plantlets with a strong tap root and 4–6 leaves into small pots filled with pre-sterilised potting mixture (sand: peat: vermiculite:: 1:2:1) and kept in a green-house with misting facility, at 80–95% humidity under low light. The new leaves were observed within 5–6 week when they were taken into bigger pots. Following this procedure they achieved 90–95% survival rate and more than 200 plantlets were transferred to the field at Indonesia. Tea plantlets with young leaves and stout roots, with a height of 4–5 cm, were taken from culture room to Hikko-trays containing pre-sterilised sand and cowdung (1:1). These Hikko-trays were then kept in poly tunnel with intermittent watering for 60 days inside indigenously developed poly house (90% survival rate). Later, they were transferred to polythene sleeves filled with black virgin soil and kept for further 1 year in the same poly-house (Mondal et al. 2001a). Following these techniques, we could produce 3000 somatic seedlings at Research and Development Department of Tata Tea Ltd, India, which has been transferred to the field.

15.3.3 Other Tissue Culture Techniques

Different other cell and tissue culture techniques have been attempted in tea that are summarised below (Table 15.6).

15.3.4 Genetic Transformation

Tea, having a woody perennial nature, has a great potential for being transgenic. After the initial standardisation of transformation protocol (Mondal et al. 1999), the healthy transgenic plants containing the marker genes were produced through *Agrobacterium tumefaciens* by Mondal et al. (2001c). The later group confirmed the stable transformation using different molecular techniques, such as polymerase chain reaction and southern hybridisation. Though transgenic plants were established under green house, yet, the stability of the transgene remains to be elucidated

Table 15.6 A brief description of some cell culture techniques used in tea

Name of technique	Objectives	Remarks	Reference
Somaclonal and gametoclonal variation	Development of mutant resistant to different stresses.	No commercial success	Rajkumar et al. (2001)
Artificial seed	Storage of propagule	Maximum 60 days storage was possible without loss of germination	Janeiro et al. 1995); Mondal et al. 2002b)
Protoplast culture	Hybrid plant production	Regeneration was not possible	Balasubramanian et al. (2000)
Anther culture	Haploid plant production	Regeneration was not achieved	Saha and Bhattacharya (1992); Raina and Iyer (1992)
Suspension culture	Secondary metabolite production	Commercially exploited	Orihara and Furuya (1990); Matsuura et al. 1991)
Cryopreservation	Long-term storage of propagule	Not exploited further either academic or commercial purposes	Kuranuki and Yoshida (1991)

as tea plants take years to flower and set seeds. Luo and Liang (2000) constructed a vector containing Bt gene {cry I A (c)}, GUS intron and NPT II and transferred to *Agrobacterium* strains LBA 4404 and EHA 105 for transgenic tea production. Using this construct they could achieve the transient expression of GUS gene in calli of putative tea plants. However, no transgenic plants were produced and transferred to the field.

Transformation through *Agrobacterium rhizogenes* is another alternative approach to transfer the gene, which in nature produce hairy roots formation. However, in in vitro, Zehra et al. (1996) were first to develop the hairy roots of tea by infecting the leaves with *Agrobacterium rhizogenes*, strain A_4. Mannopine analysis by paper electrophoresis confirmed the stable integration of transformation. Later Konwar et al. (1998), also infected basal portion of 4–6 months old in vitro tea shoots followed by co-cultivation in liquid MS medium supplemented with IBA (5 mg/l) and rifampicin (100 mg/l). After 32–45 days of culture, root initiation took place from the basal portion of 66% explants. The idea for such attempt is to enhance hardening by inducing rooting. However, tea being rich in alkaloid, commercially the technique has not been exploited to produce the secondary metabolites, which will be of immense benefit for a crop like tea.

Although, no transgenic tea plant has been produced via biolistic mediated transformation, a preliminary study on transient expression was reported by Akula and Akula (1999). Somatic embryos were bombarded with the vector p2k7 coated with gold particles (1.5–3 μm diameters). Optimisation of various factors, such as distance between the site of delivery of the microprojectile and the target tissue, helium pressure and the state of target tissue to obtain transient expression was done on the

basis of β-glucuronidase (GUS) assay after 30–40 h of bombardment. Following bombardment, the highest transient expression levels (up to 1,085 blue spots/shot) were obtained in the somatic embryos. However, further details of regeneration of transgenic somatic embryo were not mentioned.

Despite the fact that the transgenic technology has tremendous scope for tea, except developing the protocol, no transgenic plant has been developed with any agronomically useful gene so far.

15.4 Molecular Breeding

15.4.1 Morphological Markers

Morphological traits controlled by a single locus can be used as genetic markers if their expression is reproducible over a range of environments (Staub et al. 1982). Tea has been classified into three different races by morphological characters. Barua (1963) provided morphological and anatomical descriptions of the three races of tea, which were later, elaborated by Bezbaruah (1971). While leaf architect, growth habits and floral biology are important criteria used by tea taxonomists (Banerjee 1992), the bush vigour, pruning weight, periods of recovery from prune, plant height, root mass, root-shoot ratio, plucking point density, dry matter production and partitioning are considered as yield indicator in tea (Banerjee 1992). Caffeine, volatile compounds (Seurei 1996), green leaf pigmentation (Banerjee 1992), leaf pubescence (Wight and Barua 1954) are some of the parameters that have been used as potential determinants of tea quality. Despite the disadvantages, these markers are mostly adopted, frequently used by tea breeders globally (Table 15.7).

In South India, the breeding work at UPASI, Tamil Nadu started during early 1960s, which has resulted in the release of 28 clones, namely, UPASI-1 (United Planter's Association of South India) to UPASI-27 and 5 bioclonal seed stocks. Among them, UPASI-9 is considered to be drought tolerant. Recently they have also developed a clone UPASI TRF-1, which has highest yielding capacity with distinct morphological characters and quality attributes. Further to widen the genetic base, clones developed at Sri Lanka were introduced, experimented and found TRI-2024 and TRI-2025 were suitable. Development of biclonal seed stock was initiated in the late 1970s and early 1980s. Over the years, several cross combinations were tested and among them five bioclonal seed stocks, such as BSS-1 (UPASI-10 X TRI-2025), BSS-2 (UPASI-2 X TRI-2025), BSS-3 (UPASI-9 X TRI-2025), BSS-4 (UPASI-15 X TRI-2025), BSS-5 (CR6017 X UPASI-8) have been released by UPASI scientific department in 1993 (Sharma and Satyanarayana 1987).

15.4.2 Biochemical Markers

To overcome the problems of morphological markers, attention was paid for searching alternative biochemical markers. Presence and quantity of calcium oxalate

Table 15.7 Conventional markers used for germplasm characterisation/selection of breeding/assigning taxonomic position of tea

Criterion	Reference
Quantity and shape of the scleriods	Barua (1958)
Bush vigour	Barua and Dutta (1971)
Leaf geometry	Banerjee (1987)
Volatile flavour compound	Borse et al. (2002)
Leaf pose, colour, serration of the margin and angle	Eden (1976)
Chlorophyll content/photosynthesis rate	Ghosh Hazra (2001)
Quantitative changes in chlorophyll-a and chlorophyll-b and carotenoids	Hazarika and Mahanta (1984)
Epicuticular wax	Kabir et al. (1991)
Dry matter production and partitioning	Magambo and Cannell (1981)
Green leaf catechin and ratio of dihydroxylated to trihydroxylated catechin	Magoma et al. (2000)
Root length	Nagarajah and Ratnasurya (1981)
Caffeine and volatile flavour compounds	Owuor and Obanda (1998)
Leaf, floral biology and growth morphology	Sealy (1958)
Chloroform test	Sanderson (1964)
Pruning litter weight	Satyanarayan and Sharma (1982)
Anthocyanin pigmentations in young leaves	Satyanarayan and Sharma (1986)
Evenness of the flash, plucking density and recovery time of pruning.	Singh (1999)
Tarpen index	Takeo (1981)
Leaf pubescence	Wight and Barua (1954)
Phloem index	Wight (1954)

crystals in paranchymatous cells of leaf petioles, which are known as the phloem index, have been suggested to be a suitable criterion to classify the hybrids (Wight 1958). The variations in the quantity and morphology of sclereiods in the leaf lamina have also been utilised in differentiating tea taxa (Barua 1958; Barua and Dutta 1959). Considerable success has been achieved in identification of tea quality indicators (Owuor et al. 1986; Takeo 1981). These indicators have also found wide use in distinguishing the two species of tea, namely, *C. sinensis* and *C. assamica* and their respective clones (Owuor 1989). Takeo (1983) suggested a chemotaxonomic method of classifying tea clones based on a ratio referred to as the Terpene Index (T.I), which expresses the ratio between linalols and linalols plus geraniols.

Although not fully exploited, the polyphenol oxidase activity, individual polyphenols, amino acids and chlorophyll content vary between hybrids and thus can be the potential parameters in tea taxonomy (Sanderson 1964). Presence or absence of certain phenolic substances in the shoots has also been used in establishing relationship between different taxa (Roberts et al. 1958). Quantitative changes in chlorophyll-a and chlorophyll-b and four carotenoids (β-carotene, lutein, violaxanthine, and neoxanthine) was used for characterisation of Assam, China and Cambod types of tea (Hazarika and Mahanta 1984).

Total green leaf catechine concentrations and ratio of dihydroxylated to trihydroxylated catechins were used to establish genetic relationship among the 102

Kenyan tea accession (Magoma et al. 2000). Upon multivariate analysis, accumulation of the various catechins separated the tea clones in to three major and five minor groups according to their phylogenetic origins. They found that Cambod teas had the highest ratio (7:10) followed by China teas (3:5) while Assam teas had the lowest ratio (1:4). This biochemical differentiation indicates that there is potential for broadening the genetic base of mainly Assam teas in Kenya (90%) with the putative China and Cambod teas. Although accuracy is higher, however, accumulation of other chemicals is subjected to post-transcriptional modification and thus often restricts their utility (Staub et al. 1982).

15.4.3 Isozymes Markers

Genetic analysis of isozyme variation was used for cultivar identification in a wide range of plants (Ferguson and Grabe 1986; Hirai and Kozaki 1986). Similarly, in tea also, isozymes have been analysed by several workers (Hairong et al. 1987; Anderson 1994) for studying the genetic tendencies, cultivar identifications and implications in hybrid breeding. Among the isozymes, peroxidase and esterase have been the most widely studied on a wide range of tea cultivars (Chen 1996; Borthakur et al. 1995; Chengyin et al. 1992; Yang and Sun 1994; Singh and Ravindranath 1994). Isozyme analyses of tetrazolium oxidase, aspartate aminotransferase and alpha-amylase were studied among seven different tea cultivars along with three different species (Sen et al. 2000). The electrophoretic analysis revealed both the qualitative and quantitative variation in the isozyme-banding pattern among different species of tea and their clones. The tetrazolium oxidase enzyme system showed the highest variability among all the enzymes. Cluster analysis using isozyme-banding pattern produced a dendrogram, which clearly differentiated characteristics of both clones and species studied. However, in general, the application of isozyme studies in tea was limited to few enzymes with inadequate polymorphism (Wachira et al. 1995).

Wendel and Parks (1982), analysed 17 isozymes of different cultivars of *C. japonica*. They found that 15 isozymes produced 2–9 polymorphic loci while two produced 1–3 monomorphic bands. Based on the segregation of 12 loci by eight enzymes, they postulated co-dominant inheritance of single-gene traits. They also suggested that two pairs of genes are linked, that is, aspartate amino-transferase with phospho-glucomutase and 6-phosphogluconate dehydrogenase with phosphoglucomutase. In a further study, the same authors (Wendel and Parks 1983) reported isozyme variations at 15 loci from 12 enzymes with 205 genotypes of *C. japonica*. All loci were polymorphic and a total of 64 alleles were detected. Peroxidase and 6-phophogluconate dehydrogenase (6-PGDN) isozyme were also used to differentiate between varieties of *C. sinensis* and *C. japonica* (Ikeda et al. 1991). They concluded that alcohol dehydrogenease isozymes in *C. japonica* are encoded by two genes *Adh*-1 and *Adh*-2. Both loci are expressed in seeds and their products are randomly associated with intragenic and intergenic dimmers. Electrophoresis of leaf extracts produces only the products of *Adh*-2. Formal genetic analysis indicated that the

two *Adh* loci are tightly linked. Most segregations fit the expected Mendelian ratios but in some families distorted segregation was also observed at *Adh*-1, *Adh*-2 or both (Wendel and Parks 1984). Starch gel electrophoresis was used to score allelic variation at 20 loci in seeds of *C japonica* collected from 60 populations distributed throughout Japan. In comparison with other plant species, the genetic diversity within the population is very high, that is, 66.2% of loci were polymorphic per population, which gave an average mean number of 2.16 allele per locus. They also reported genotypic proportions at most of the loci in majority of all the population and found a good fit of the Hardy–Weinberg expectations (Wendel and Parks 1985).

15.4.4 DNA Markers

Limited number of loci of isozyme showed the lesser polymorphism but with the advancement of molecular biology such efforts were shifted towards various DNA based markers. However, due to widespread cultivation of clonal tea by elite planting material, the genetic diversity is diminishing gradually. Therefore germplasm characterisation at molecular level of tea will help: (1) varietal improvement of tea for agronomically important character, (2) to preserve the intellectual property right of tea breeder, (3) identification of individual tea cultivar by making a molecular passport, (4) prevention of duplicate entry of different genotypes in tea gene pool, (5) efficient selection of the varieties for hybridisation program, graft compatibility in composite plant production, and so on and (6) taxonomic classification of tea genotypes on the basis of molecular markers. Since ever the work of Wachira et al. (1995) on RAPD analysis of tea germplasm, a number of markers have been studied till today (Table 15.8).

15.4.4.1 RAPD Markers

Since discovery, random amplified polymorphic DNA (RAPD) marker (Williams et al.1990) is being used for a number of areas in plant taxonomy. At present it is the most preferred DNA markers due to (1) greater speed, (2) easy-to-perform and (3) non-requirement of radioactive materials, and so on. In tea and other species of *Camellia*, a considerable amount of work has been carried out which are summarised below.

Wachira et al. (1995) were the first to characterise 38 different cultivars of Kenyan tea. Based on the analysis of 23 primers, a dendrogram and principal coordinate (PCA) was constructed based on average linkage cluster analysis. Except a few genotypes, the molecular marker was able to discriminate different varieties of Assam, Cambod and China tea. In a preliminary study, Tanaka et al. (1995) used several 10-mer and 12-mer primers to detect variation among Korean, Japanese, Chinese, Indian and Vietnamese tea. Among all primers OPF-2 was found to be the most polymorphic. The study concluded that Korean tea has undergone little genetic diversification after introduction from China. On the contrary, Japanese tea showed

Table 15.8 Summary of different molecular markers used in tea and other *Camellia*

Country	Name of the marker	Objectives	Size of the population	Reference
India	RAPD	Characterisation of micropropagated tea plants	18 randomly selected tissue culture plants.	Mondal and Chand (2002)
	RAPD	Genetic diversity	25 genotypes of tea and 2 different *Camellia* species	Mondal (2000)
	RAPD	Inexpensive methods of isolation DNA	20 genotypes of tea and 8 other highly content phenolic genera	Mondal et al. (2000a)
	ISSR	Genetic diversity	25 genotypes	Mondal (2002b)
	AFLP	Genetic diversity	32 genotypes	Paul et al. (1997)
	AFLP	Genetic diversity	49 genotypes	Balasaravanan et al. (2003)
	AFLP	Genetic diversity	29 genotypes	Mishra and Sen-Mandi (2001)
	AFLP	Genetic diversity	32 genotypes	Rajasekaran (1997)
	RFLP	Species-specific PCR primers were developed from intergenic spacer regions of 5S ribosomal RNA genes	Used successfully in the detection of adulteration of cashew husk in 10 different tea samples.	Dhiman and Singh (2003)
	RFLP	Organisation of 5S ribosomal RNA genes in tea	8 genotypes	Singh and Singh (2001)
Japan	STS-RFLP	Authentication of made tea sample	46 cultivars	Kaundun and Matsumoto (2003b)
	SSR	Genetic diversity	518 ecotypes of *C. Japonica* collected from different location	Ueno et al. (2000)
	SSR	Development and characterisation of microsatellites	8 pairs of microsatellite developed	Ueno et al. (1999)
	RFLP	Genetic analysis	30 genotypes	Matsumoto et al. (1994)
	RFLP	Genetic analysis of PAL gene	29 cultivars and 463 promising breeding line.	Matsumoto et al. (2002)
	RAPD	Identification of true crosses	2 cultivars and F_1 progenies	Tanaka and Yamaguchi (1996)
	CAPS	Species specific probe	50 genotypes	Kaundun and Matsumoto (2003)

Table 15.8 (continued)

Country	Name of the marker	Objectives	Size of the population	Reference
China	SSR	Heterologus nuclear and chloroplast microsatellite probe	24 genotypes	Kaundun and Matsumoto (2003)
	RAPD and AFLP	Genetic analysis	44 genotypes of tea and 4 wild relatives	Wachira et al. (2001)
	RFLP	Genetic analysis using PAL probe	29 cultivars and 463 promising breeding line	Matsumoto et al. (2002)
	RAPD	Genetic analysis of tea and related genera	23 species	Chen and Yamaguchi (2002)
	RAPD	Genetic analysis	24 wild tea genotypes	Chen et al. (2002a)
	RAPD analysis	Molecular phylogeny	24 species	Chen et al. (2002b)
	RAPD And RFLP	Rapid methods for isolation of DNA	7 genotypes	Chen et al. (1997)
	RAPD	Genetic analysis	7 genotypes	Chen et al. (1998a)
	RAPD	Genetic analysis	15 genotypes	Chen et al. (1998b)
	RAPD	Genetic analysis	5 genotypes	Chen et al. (1999)
Kenya	RAPD	Genetic diversity	38 genotypes	Wachira et al. (1995)
	RAPD	Species-specific product and diagnostic analysis for gene introgression	8 genotypes of tea and 20 *Camellia* species	Wachira et al. (1997)
	RAPD and AFLP	Genetic linkage map	90 genotypes of F_1 progeny	Hackett et al. (2000)
South Africa	RAPD	Genetic diversity	5 genotypes	Wright et al. (1996)
Taiwan	RAPD and ISSR	Genetic analysis	37 genotypes	Lai et al. (2001)
South Korea	RAPD	Genetic analysis	6 genotypes	Kaundun and Park (2002)
	RAPD	Genetic analysis	27 genotypes	Kaundun et al. (2000)
	AFLP	Genetic analysis	37 genotypes	Lee et al. (2003)
Portuguese	RAPD	Genetic analysis	71 genotypes	Jorge et al. (2003)
USA	Sequence analysis	Evolutionary study	30 different taxa including tea	Prince and Parks (1997)
	Sequences analysis	Phylogenic relationship	19 different species including tea	Prince and Parks (2000)
	Sequence analysis	Evolutionary study	35 different taxa including tea	Prince and Parks (2001)

a closer relationship with their Chinese and Indian counterpart, which reveal the fact that tea in Japan might have been brought from China and India.

In another study, 25 important Indian tea cultivars and two ornamental species were examined by Mondal (2000). Out of the 40 random 10-mer primers, 11 generated polymorphic banding pattern with a range from 7 to 21 per genotype. Out of the total 154 bands, 138 were polymorphic resulting in a high genetic variability of 95.2%. Shannon's index of diversity was used to partition the total phenotypic variation into intra and inter-population components. On an average, 57% within and 43% between population variability was revealed. A dendrogram was constructed on the basis of band sharing, separated the population into three clusters, that is, China, Assam and Ornamental type. The China type was more variable than the Assam type. The PCA revealed that the Chinary clones are more dispersed than Assam clones, thus suggesting that a greater genetic diversity exists among Chinary clones. The results showed that RAPD markers could be used effectively to distinguish and characterise Indian tea germplasm.

In a separate study, the diversity of 27 tea accessions from Korea, Japan and Taiwan was examined with 50 RAPD primers. Diversity was greatest within the Korean group followed by Taiwan and Japan. The relatively high diversity observed in Korea might reflect the larger genetic base of its plantations while the low diversity in Japan could be explained by the long and intensive tea selection programme. A dendogram based on the unweighted pair group method analysis (UPGMA) along with Jaccard's distances and multivariate factorial correspondence analysis clustered the tea accessions into two main groups, that is, Taiwan and the Korean cultivars. This suggests that the Taiwan tea studied here might have a different origin from that of Korean and Japanese tea (Kaundun et al. 2000)

15.4.4.2 ISSR Markers

Inter-simple sequence repeat (ISSR) has been used for genetic characterisation of various plant species (Tsumura et al. 1996). Because of greater length of ISSR primers, they show greater repeatability and stability of map position in the genome while comparing genotypes of closely related individual (Zietkiewicz et al. 1994).

Twenty-five diverse tea cultivars were analysed using the simple sequence repeat anchored polymerase chain reaction (SSR-anchored PCR) or Inter SSR-PCR (ISSR). Based on the polymorphism, 12 out of 45 primers were chosen for final study. These 12 primers amplified a total of 130 bands, of which 108 (84%) were polymorphic. A dendogram was constructed using UPGMA method (Fig. 15.5) and revealed three distinct clusters of Cambod, Assam and China type, which concurs with the known taxonomical classification of tea. These results suggest that the ISSR-PCR method can be used potentially for genetic fingerprinting and taxonomic classification of tea genotypes (Mondal 2002b).

Tea is also an important crop of Taiwan and most of the cultivated tea in that country were introduced from China and India though some native wild teas are distributed in the mountains of Central and southern Taiwan. Lai et al. (2001) carried out RAPD and ISSR marker analysis of 21 clones of China, 3 clones of Assam,

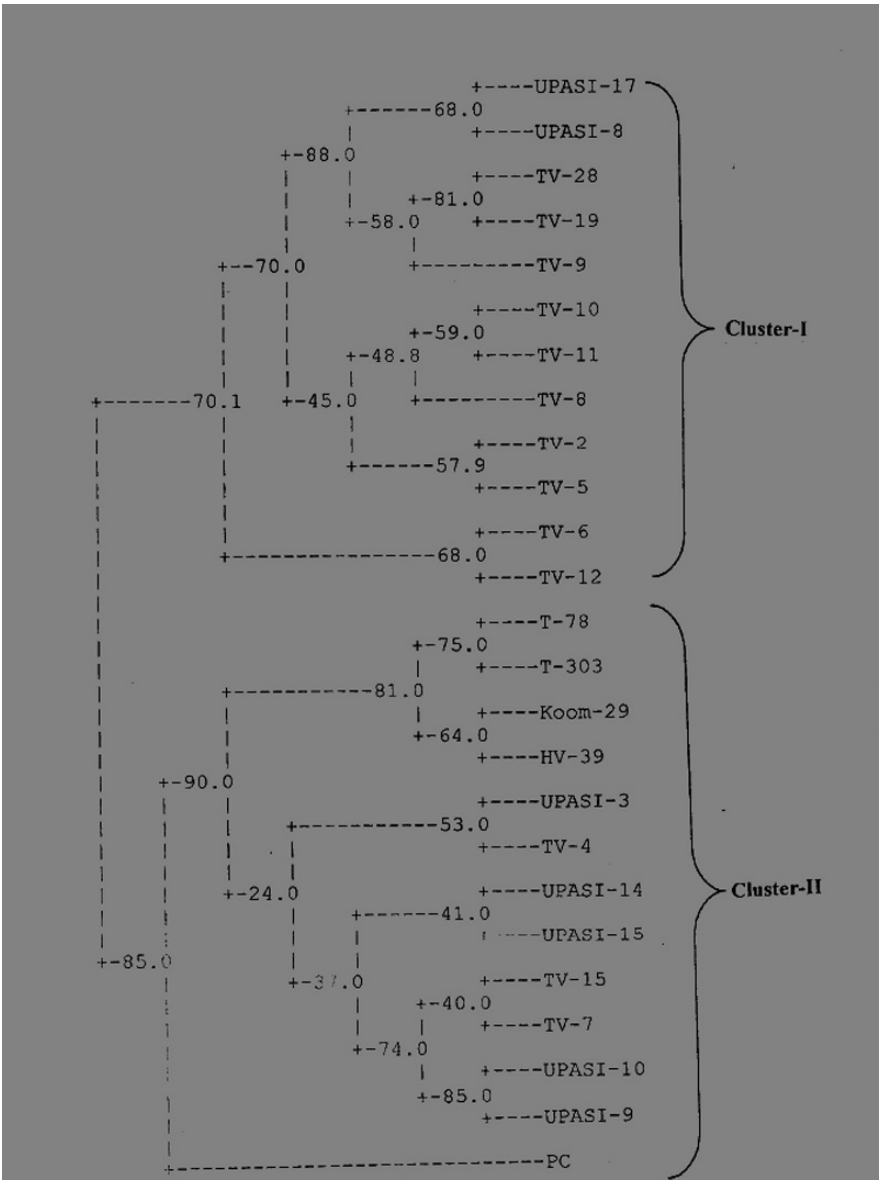

Fig. 15.5 UPGMA derived dendrogram illustrating the relationship among the 25 important Indian tea cultivars as inferred by ISSR-PCR analysis

7 hybrids clones between China and Assam tea and 6 individual samples of native Taiwanese wild tea. A total of 53 and 56 polymorphic RAPD and ISSR markers were scored. The results of cluster analysis based on RAPDs revealed that three major groups could be recognised, that is, cultivars of China tea and the cultivars developed in Taiwan from hybridisation and selection and native Taiwanese wild

tea. The native Taiwanese wild teas were, however, most distant in the clustering tree. In the ISSR dendogram, Taiwanese native wild teas clustered closely with Assam tea then with China tea and the Taiwanese hybrid cultivars. The population gene diversity of the native wild tea was found to be the highest among the three populations studied.

15.4.4.3 RFLP Markers

Restriction fragment length polymorphism (RFLP) has been used to investigate genetic diversity in cultivated plants and wild relatives (Tanksley et al. 1989). In tea, Matsumoto et al. (1994) cloned the PAL gene using the rice cDNA as heterologus probe and used for studying the genetic variation of Japanese green tea using RFLP technique. On the basis of the numbers and molecular weight of the detected fragments they classified Japanese green tea cultivars into five groups that have different routes of origin. All the Assam hybrids used in their study could be distinguished from Japanese green tea cultivars on the basis of this grouping.

Furthermore, the inheritance of PAL gene in tea was investigated. It was concluded that PAL gene was a single copy per haploid genome and was inherited as a single gene according to the Mendelian ratio of 1:2:1. The work was further extended to the Japanese tea with green tea cultivars using PAL as DNA marker. The main DNA fragments detected by RFLP analysis, which were named as A, B, and D were inherited as multiple allelic genes at one locus. They concluded that most of the cultivars belonging to the AA genotype group had been selected from local tea plants about 50 years ago. On the other hand, all the cultivars in the BD genotype group were either from cultivar Yabukita or its hybrids. The other genotypes (AD, AB and DD) included cultivars selected from local tea plants and their hybrids. To investigate the reason for the absence of any BB genotypes, allelic frequency of all genotypes were studied and found that B was the least among the three with a frequency of 0.08. Hence, the chance of occurrence of BB genotype is (0.08×0.08) 0.0064. This is the reason why BB genotype was not found among the population (Matsumoto et al. 2002)

RFLP technique was also applied to identify the processed tea sample. In Japan, tea produced from 'Yabukita' fetch better realisation in the market and hence there is a trade tendency to adulterate the low-grade tea with Yabukita, which is difficult to detect either visually or through tester tong. To solve the problem, Kaundun and Matsumoto (2003b), employed the sequence tag site (STS)-RFLP using the sequence information of three genes, namely, phenyl-ammonia lyase, chalcone synthase and dihydroflavonol 4-reductase. The restriction digestion of the specific amplification suggested the authentication of 46 samples.

15.4.4.4 AFLP Markers

Amplified fragment length polymorphism (AFLP) being a reliable and the most robust DNA marker, can detect more number of polymorphisms than RFLPs or RAPDs (Vos et al. 1995). Thus AFLP markers offer an opportunity to perform detailed genetic studies in closely related population (Meksen et al. 1995).

In tea, Paul et al. (1997) were the first to employ AFLP markers to detect diversity and genetic differentiation of 32 tea clones comprising Indian and Kenyan origin. Five enzyme-primer combinations revealed 73 unambiguous polymorphic bands. The dendogram constructed on the basis of shared fragments, grouped into three known types, that is, Assam, China and Cambod, which generally concur with the existing knowledge on the biosystematics of tea. Further, the PCA revealed that Assam clones from India and Kenya clustered closely indicating a common ancestry.

In the same year, Rajasekaran (1997) reported the AFLP analysis of 42 tea clones, which includes 23 UPASI, 17 popular South Indian estate selected clones and two Kenyan tea clones. He concluded that 90% of the UPASI clones are inbred and thus unsuitable for commercial cultivation. Importantly, three clones namely, SMP-1, UPASI-15 as well as TRI-2025 clustered together, all of which were tolerant to blister blight disease, whereas highly susceptible clones of the same diseases, such as UPASI-4, UPASI-7 and UAPASI-12 grouped together. Therefore, he concluded that AFLP could be utilised successfully for developing the markers related to blister blight diseases resistance.

Mishra and Sen-Mandi (2001) did AFLP analysis of 29 popular Darjeeling tea cultivars. Using eight primer pair combinations, 677 bands were observed, among which 469 were polymorphic. The dendogram obtained, clearly divided the clones into three groups,that is, Assam, China and Cambod type. All the cultivars were grouped according to the known conventional taxonomic classification barring two clones, T-246 and T-135, which are known as Assam type but in dendrogam they grouped separately. Thus, they suggested that these two clones might have originated through extensive cross breeding between species within the genus *Camellia*.

15.4.4.5 SSR Markers

Simple sequence repeats (SSRs), known as microsatellites are tandemly repeated DNA sequence motifs (usually 2–5 bp long) that are highly polymorphic in plant genomes (Wu and Tanksley 1993). Due to their hypervariability, relative ease of scoring by PCR, co-dominant nature and high reproducibility, they are now considered to be the most reliable genetic marker.

Ueno et al. (1999) were pioneer to develop the SSRs from *C. japonica*, a closely related species of tea. Out of the total 339 RAPD amplifications, 21 were found to contain microsatellite repeats. Finally, four primer pairs were developed which yielded single-locus polymorphic amplification product. Using these primer pairs, 53 *C. japonica* ecotypes were genotyped and population genetic parameters were calculated. The following year, Ueno et al. (2000) investigated the spatial genetic structure of *C. japonica* using these four microsatellite primers. Spatial distribution of individuals was also assessed to obtain an insight into spatial relationships between individuals and alleles. Morisita's index of dispersion plotted 518 individuals of *C. japonica* in a single clump and Moran's *I* spatial autocorrelation coefficient revealed weak genetic structure, indicating a low level of allele clustering among the individuals.

However, being costly and technically demanding, it is difficult to develop the microsatellite always. Alternatively, often microsatellite markers of closely related species are used. Using the information of Ueno et al. (1999), Kaundun and Matsumoto (2002) attempted to transfer nuclear microsatellites isolated from *Camellia japonica* and chloroplast microsatellite detected in tobacco into tea. They subsequently used them to fingerprint and estimate the level of polymorphism and differentiation between the two main Assam and China species. The four nuclear microsatellite primers tested in tea amplified a DNA fragment of approximately the same length as of *C. japonica*.

15.4.4.6 CAPS Markers

Cleaved amplified polymorphic sequences (CAPS) are alternative molecular markers, which combine both PCR and RFLP techniques. The techniques require minute amount of DNA and simple electrophoresis system to reveal polymorphism. CAPS markers were successfully applied to a number of crops and forest species for which extensive nucleotide information is available. The advantages of these markers are development of mapped cDNA clones that represent expressed genes.

The genetic diversity of tea in both taxons was investigated based with CAPS analysis of Phenylalanine ammonia-lyase *(PAL)*, Chalcone synthase *(CHS2)* and Dihydroflavonol 4-reductase *(DFR)* gene (Kaundun and Matsumoto 2003a). These three genes involve in catechin and tannin biosynthesis, hence directly responsible for tea taste and aroma. The critical factor in development of CAPS markers is the right choice of PCR primers targeting a single locus at a time, in order to ensure reliable results. This was facilitated here because both PAL and DFR exist in one and two copies, respectively, in tea. The CHS gene is present in three copies but with sufficient sequence difference among them, allowing specific amplification of CHS2 only. Based on their analysis it was revealed that China type was more variable than Assam type and that a higher proportion of over all diversity resided within varieties as compared to between varieties. Even though no specific DNA profile was found for either tea varieties following any single PCR-RFLP analysis, a factorial correspondence analysis carried out on all genotypes and markers separated the tea sample into two distinct groups according to their varietal status. This reflects the larger differences between the two taxa in their polyphenolic profiles. Thus, STS-based markers developed will be very useful in future mapping, population genetics and fingerprinting studies of this important crop species and other *Camellia* species. This work also opens the way for correlative analysis between newly established tea genotypes based on phenylpropanoid genes and chemotypes, mainly polyphenolic.

15.4.4.7 Genomics

Genomics and its global expression profile (proteomics) offer an additional advantage for rapid identification of genes and pathway to control important plant traits. Multigenic characters, such as abiotic stress, drought and frost, and so on being the major production constrain in tea cultivation can be studied in-depth. However, the

prerequisite of any genomics study is the development of large amount of Express Sequence Tag (EST) and very recently with the pioneer initiative of Tea Research Institute, Hangzhou, China 1,257 number of EST has been developed. These ESTs have been prepared from a cDNA library of young tealeaves and available in the NCBI database. However, prior to this, work on gene expression was initiated by Takeuchi et al. (1994a) when they isolated cDNA of Chalcone synthase (CHS) gene from a Japanese green tea cultivar 'Yabukuta'. From then a large number of full length genes have been reported in tea.

15.5 Conclusion and Prospects

So far genetic improvement of tea is mainly contributed by conventional breeding, however, in the recent two decades great attempts has been made to intervene some of the conventional breeding through biotechnology. It is noteworthy to mention that since the work of Forrest (1969), the pioneer of tea micro-propagation, the technique has been worked out well although commercially not exploited. This is perhaps due to the fact that vegetative propagation techniques are well established and cost effective. Certain areas, such as somatic embryogenesis, cell culture for secondary metabolism has been standardised. Despite the fact, the transgenic technology has tremendous scope for tea, no transgenic plants have been cultivated commercially so far. Among the many reasons, few could be (1) tea plant is not easily amenable for *Agrobactrium* infection, (2) non-availability of generic protocol, which can be applied for wide range of varieties of tea plant, (3) being woody perennial, production of transgenic tea is time consuming and hence private funding is less. However, it is evident now among the different techniques *Agrobacteium tumefacians*-mediated transformation has been attempted with different groups but transgenic tea is yet to be commercialised. On the other hand, though *Agrobacteium rhizogenes* transformation in tea has been demonstrated, the technique has not been exploited commercially to produce the secondary metabolites so far which will be of immensely useful for a crop like tea. Contrary to the above techniques, work on particle bombardment is in very initial stage. It is noteworthy to mention here perhaps transgenic tea has an unique advantage, as during processing green tea leaves are exposed to a temperature of 120°C at drying step in which toxins will be destroyed, which is critical for GM foods of some agricultural crops globally. A detail study through international collaboration by a common consortium will be the right approach for production of transgenic tea in the long term.

Till now, several DNA markers have been used to make fingerprints, which need to be documented systematically and should be made available for public use to preserve the IPR of tea breeders. Although in several ways tea molecular biology work can be directed, yet priority should be given:

- to undertake a massive germplasm characterisation of tea germplasm across the world through a common 'Tea germplasm characterisation consortium', which already exist for several similar crops.

- DNA marker need to be identified to do early selection at nursery stage for various biotic and abiotic stresses, which will revolutionise tea breeding where works suffer due to the lack of selection criteria and long gestation periods, and so on.
- The ultimate work of DNA markers that has just been initiated recently (Hackett et al. 2000) for construction of genetic linkage map, which need to be focused immediately and should be correlated with the application of functional genomics and proteomics.
- To generate and characterise the ESTs of tea.

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Chapter 16
Genetic Improvement in Cocoa

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16.1 Introduction

Cocoa, *Theobroma cacao* L., is a perennial crop, originally from the South and Central America and normally cultivated in the tropics from latitude 18° N to 15° S, mainly in small farms, under the shade of native trees. Although it has been explored by the Mayans and Aztecs since pre-Colombian times, only in the last 50–60 years it has received some attention in respect to breeding. The cocoa beans are the major ingredient for the cocoa industry, for the manufacturing of chocolate and derivatives, cosmetics and medicines. Cocoa is cultivated commercially in South and Central Americas, Caribbean, Africa, Asia and in some Pacific Islands. But most of the cocoa currently produced in the world (around 70%) comes from West African countries, especially Ivory Coast, Ghana and Nigeria.

The evidence of cocoa as a domesticated crop comes from archaeological findings in Costa Rica indicating that cocoa was already used as a drink by the Mayans as early as 400 BC. In the 14th century, the Aztecs regarded the cocoa as the food of the Gods, placing much emphasis on the sanctity of cocoa. The spread of cocoa around the world began during the Spanish colonialism (Bergman 1969). Christopher Columbus was the first European to come in contact with cocoa, who reached Nicaragua in 1502, searching for a sea route to India (Bergman 1969; Cheesman 1944). At that period, cocoa was already an important tree to the indigenous people. Cocoa beans were used as currency in some parts of Central America. It was Hernan Cortés, leader of an expedition in 1519 to the Aztec empire, who returned to Spain in 1528 bearing the Aztec recipe for *xocoatl* (chocolate drink) with him. The drink was initially received unenthusiastically and it was not until sugar was added that it became a popular drink in the Spanish courts. Its formula was kept as a secret to be enjoyed only by nobility. Eventually, the secret was revealed and the drink's fame spread to other lands (Purseglove 1968). As the cocoa drink had become so appreciated, the European demand for the product also increased at that time, as

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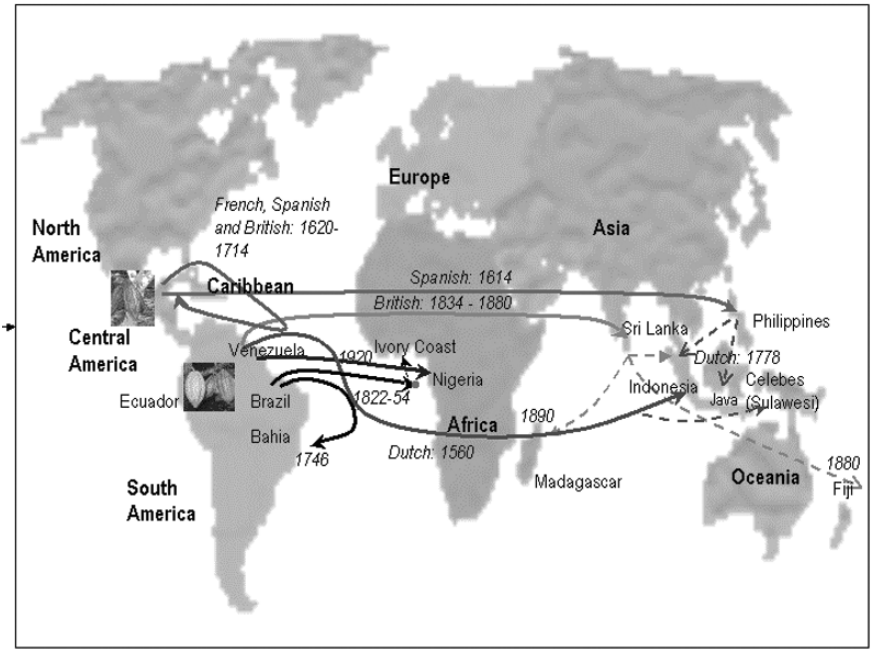

Fig. 16.1 World wide distribution of cocoa (*See* Color Insert)

so the interest in other new lands for expanding cocoa plantings. The spreading of cocoa around the world is shown in Fig. 16.1 with the main routes taken by Spaniards, French and Dutch.

There were attempts to satisfy Spanish domestic demand by planting cocoa in the Spanish colonies like the Dominican Republic, Trinidad and Haiti. In 1560, the cocoa from Caracas, Venezuela, was introduced in the Island of Sulawesi, in Indonesia. In 1600, the Spanish also introduced cocoa in Philippines. In 1615, the French became aware of the use of cocoa, a century after the first discovery of the chocolate drink by the Spanish court. By the mid-1600s, the chocolate drink had gained widespread popularity in France. The Spanish princess Anna of Austria married French King Louis XIII who introduced, among other Spanish customs, the drinking of chocolate at the French court. There was increasing interest by the Europeans for new lands for the cultivation of cocoa. The French introduced cocoa in many locations, such as the islands of Martinique and St Lucia in 1660, Dominican Republic in 1665, Guyanas in 1684 and Grenada in 1714. The Caribbean Islands of Martinique became one of the major cocoa producers by 1680. The English took this crop to Jamaica by 1670; and, the Dutch took over plantations in the Curaçao islands in 1620 and introduced cocoa from Philippines to Indonesia and Malaysia in 1778. Cocoa was introduced to Bahia, in 1746, with seeds coming from Pará, a northern State of Brazil. With the increase in demand for chocolate in Europe, more areas were required for expanding the cocoa plantings. Thus, the cocoa was taken

to Africa in the beginning of the eighteenth century with the introduction of the *Amelonado* cocoa from Brazil to the islands of Príncipe (1822), São Tomé (1830) and Fernando Pó (1854). Later on, cocoa was introduced in the African continent, when it was taken from the Island of Fernando Pó to Nigeria (1874) and Ghana (1879) and, in 1905 cocoa arrived to the Ivory Coast, presently the world's largest cocoa producer. In Cameroon, cocoa was introduced during the colonial period of 1925–1939 (Paulin and Eskes 1995).

There are also reports on two introductions of *Trinitario* cocoa from Trinidad to Sri Lanka in 1834 and 1880. And later, this variety was introduced to Singapore, Fiji, Samoa, Tanzania and Madagascar, from Sri Lanka. In Java, the failure of the coffee crop in 1880 encouraged the farmers to grow cocoa (Paulin and Eskes 1995; Purseglove 1968). Cocoa occurs naturally in the South of Mexico to Bolivia and also in the Brazilian Amazon Forest. Due to the large genetic diversity of species observed, it was suggested that the cocoa tree originated in the Upper Amazon, in the zones of the confluence of the rivers Solimões, Putumayo and Caquetá. In spontaneous distribution, the cocoa tree is found in the lower stratum of the forests, in glades and on the banks of the great rivers, where high temperature and high humidity prevail (Cheesman 1944; Cuatrecasas 1964; Bartley 2005).

16.2 Botany

The species *Theobroma cacao L.* belongs to the *Malvaceae* family, genus *Theobroma* that comprises 22 species (Alverson et al. 1999; APG II 2003). Of these, only *T. cacao* (cocoa) and *T. grandiflorum* (cupuassu) are explored commercially (Cuatrecasas 1964). In wild conditions cocoa trees may reach up to 20 m of height, but under cultivation they usually reach heights of around 5 m. The seed cotyledons represent the part of economical importance. This species presents cauliflory, a type of inflorescence where the flowers arise in structures denominated floral cushions, along the trunk and secondary and tertiary ramifications. The flowers are hermaphrodite and pentamerous, having petals, sepals, stamens and staminodes. Barriers formed by the staminodes and petals isolate the reproductive organs. The pollen is sticky, forming small clots that favour their adherence to the body of insects.

Cocoa is an allogamous plant, with a high frequency of cross-pollinations. The flower structure makes self-pollinations difficult because the petals involve the anthers and a crown of staminodes surrounds the stigma. Usually, small midges of the *Forcipomiya* genus pollinate the cocoa tree, although other small insects have been reported as eventual pollinators. After pollination, the pods start developing and they will reach maturity in approximately 5–6 months, depending on temperature and variety. The cocoa pod is characterised as an indehiscent drupe that varies in color, shape, size and husk thickness and smoothness (Fig. 16.2). The number of seeds in the pods is also variable and is defined by the number of ovules in the ovary. In average, it is necessary that more than 15 ovules be fertilized to generate a pod. Nevertheless, it is very common to find plants in cocoa populations that produce parthenocarpic (seedless) pods. The seeds are recalcitrant and present large variation

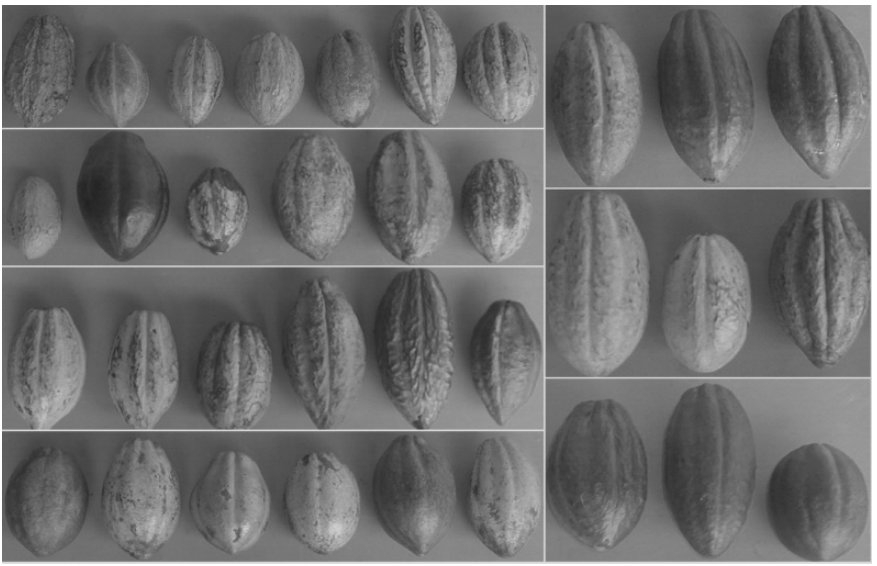

Fig. 16.2 Pod variation in color, shape and size (*See* Color Insert)

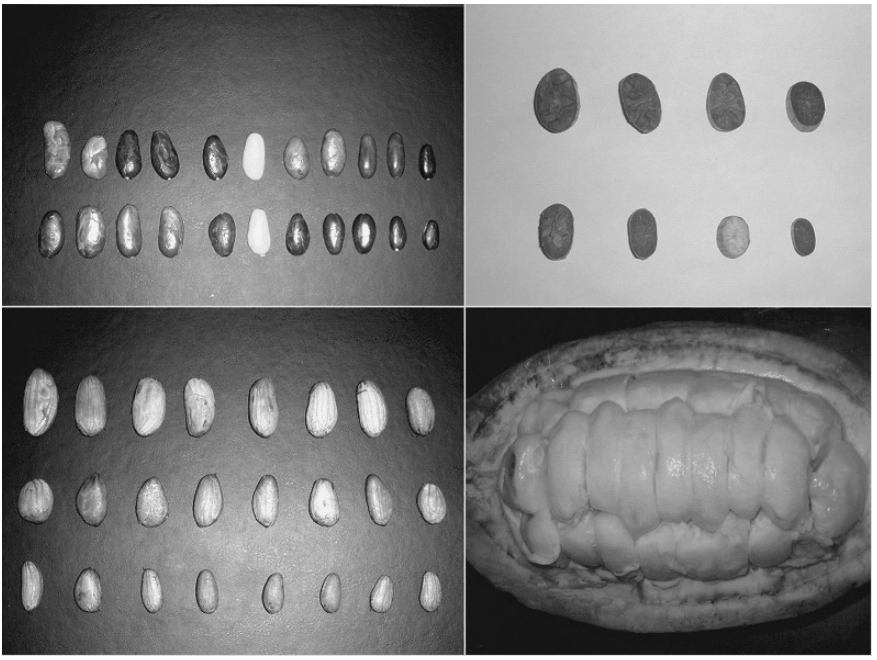

Fig. 16.3 Examples of seed variation in color, size and shape (*See* Color Insert)

in size, shape and color (Fig. 16.3). The seed color is defined by the quantity of anthocyanin pigments and can vary from white to dark purple. They are involved by mucilage that contains a germination inhibitor. Mucilage pulp can be extracted and explored economically.

16.3 Genetic Status

Cocoa is a diploid species with 20 chromosomes in the somatic cells ($2n = 20$) and a haploid genome size with around 0.43 picograms (Figueira et al. 1994), which it is just twice as much as the model species *Arabidopsis thaliana*. A wide genetic variability can be found for most traits, especially for those with agronomic importance. Bartley (2005) in his book "*The Genetic Diversity of Cacao and its Utilization*" gives an ample discussion on this matter.

16.4 Genetic Incompatibility

Sexual incompatibility in cocoa seems to be controlled by the gene *S* and its multiple alleles. The self-incompatibility in flowering plants, such as cocoa, is nothing else but a biochemical process of recognition and rejection that impedes the self-fertilization (syngamy). F. Pound observed this phenomenon in 1932 for the first time in cocoa. Later on, other investigators studying the genetic mechanism of the self-incompatibility in cocoa (Knight and Rogers 1953, 1955 and Cope 1958, 1959, 1962) showed that an incompatible pollination does not result out of the inhibition of the germination of the pollen or of the growth of the pollen tube. What happens is a failure in the fusion of the spermatic nucleus of the pollen grain with the egg cell in a number of ovules after an incompatible pollination, leading to the flower abortion. The self-incompatibility system in cocoa is unique, once the expression of the gene S (recognition gene) appears to occur about when the pollen tube enters in contact with the ovule, thus resulting in the rejection reaction and consequently, in the abscission of the flower, but not in deposition of callus in the pollen tube (de Nettancourt 1977). The self-incompatibility genetic system in cocoa is of the gametophytic-sporophytic type, but in the literature, examples of ovarian inhibition exists and pseudo-compatibility, that is, the occurrence of self-fertilization in certain self-incompatible plants under special physiological and environmental conditions.

The self-incompatibility mechanism in cocoa is not yet entirely elucidated and deserves further investigation (de Nettancourt 1977, 2000). There are monoclonal cocoa plantings of single self-incompatible clones, such as TSH-565 or TSH-1188 that bears pods inexplicably. What else could be involved in the control of this phenomenon? How can environmental conditions influence on the self-incompatibility response? These are questions yet to be answered. The interactions between pollen grain and the pistil (stigma and style) certainly may be involved. The development of the tube can be inhibited either in the style or in the stigma, or even, in the ovary. There are other studies, for instance, showing that the recognition of

self-compatibility occurs before the contact of the pollen with the ovule. Aneja et al. (1994) observed that the pollen grain did not germinate in the stigma of IMC-30, a self-incompatible clone. However, the incompatibility reaction was inhibited with the application of CO_2 in the self-pollinated flowers. Thus, the self-incompatibility mechanism in cocoa seems to act during two stages, that is, the first, during the germination and development of the pollen grain and the second during the fusion of gametes.

In cocoa, the floral abscission can be a consequence of the rejection reaction that is preceded by the recognition of hormonal changes leading to the abscission. Abscission is a process of hormonal regulation that involves the organ of the plant and the abscission area. Ethylene and auxin have a primary role in the flower abscission. The former acts as the main promoter of the abscission while the later acts either inhibiting or increasing the production of ethylene and, consequently, preventing or accelerating the abscission reaction. The concentration of abscisic acid increases in the abscission tissues and participates in the process. The incompatibility reaction occurs in two steps: first, with an increase of the amount of abscisic acid in response to pollen-stigma interaction; and second, with changes in the levels of auxin and ethylene, after the contact of the pollen tube with the ovule, establishing the hormonal conditions that determines the flower abortion. The abscission seems to occur whenever high levels of ethylene and abscisic acid are present in the flowers, what is commonly observed in self-pollinated incompatible flowers. Thus, the abscission process can be delayed or even inhibited with the application of CO_2 or synthetic auxins or of any other compounds that are able to increase the level of auxins or to reduce the levels of abscisic acid and/or ethylene. Molecular markers studies are in progress in Brazil and France with the objective of identifying markers associated to this character, what will make possible, early selection of self-compatible genotypes.

16.5 Populations

The natural cocoa populations are comprised of three major groups: the Amazon *Forasteros*, the *Criollos* and the *Trinitarios*. The Amazon *Forasteros* are subdivided according to their origin as Lower and Upper Amazon. They present green pods when immature, seeds intensely pigmented and show a large variation in shape, size and number of pods per plant and seeds per pod. In this group many clones are used in breeding programmes, including those resistant to the main diseases and plagues of cocoa. Besides that, the *Forasteros* are the most widely planted cocoa-type in the world.

The *Criollos*, originally cultivated in Central America, are characterised by having big seeds, with a round transversal section and the colour of the cotyledons varying from white to light violet and immature fruits with colours varying from green to red with rugosity. In this group there are populations that excel others for producing a fine chocolate of excellent quality and flavour, as the *Nacional* from Ecuador and the *Porcelana* from Venezuela. The *Trinitarios* are natural hybrids

between *Forasteros* and *Criollos*. They inherited traits of these two populations and are characterised by presenting a wide variation for most morphologic traits, as pod color (from green to red), thickness of pod husk, seed number, seed and pod size, and so forth.

16.6 Germplasm Collections

The cocoa collections represent the base for the cocoa breeders to develop improved varieties for commercial plantings. In these collections the genes are kept as live plants that can be considered as 'protecting shields' for the development of new varieties whenever new pests or disease eventuality occur. Different types of cocoa and related species and genera collected in areas of natural occurrence or derived either from genetic improvement programmes or from farm populations are gathered in these collections.

The most important cocoa germplasm collections are well distributed around the world, giving support to various breeding programmes. In the Americas, for example, there are two collections in Trinidad: the international cocoa collection at the Cocoa Research Unit (CRU) and the other maintained by the Ministry of Agriculture. In Costa Rica, there is also another quite large international cocoa collection in CATIE, Turrialba. These two international collections are complementary. According to Bekele (www.uwi.tt/cru/fb-hocp-ncnp.pdf), in the collections of Trinidad are conserved cocoa accessions, such as the Imperial College Selections (1930–1934); the Upper Amazon collections made by F. J. Pound (1937–1942), with specific interest in gathering cocoa germplasm with resistance to witches' broom; the germplasms collected in 1952 by the Anglo-Colombian cocoa expedition in the Colombia Territory, that included wild and cultivated *Theobroma cacao*, other species of this genus and also the allied genus *Herrania*; Ecuadorian collections (1969, 1973); LCT-EEN collection (1980–1985); The Cocoa Research Unit's local Germplasm Collection (1991); Caribbean collections (Granada (1940s), Dominica, Martinique, Guadeloupe (1986–1990)); French Guiana collection (1995); Belize collection (1992, 1994, 1996); and Ecuadorian collection (2001). As these expeditions are quite costly, the best way to collect wild cocoa germplasm would be by seeds. A sample of seeds from a specific region may represent more adequately the natural variability of the population. In CEPLAC, Brazil, there are two large cocoa germplasm collections. The largest one is established in the North of Brazil, in the State of Pará. It is represented mainly by wild accessions collected in the Brazilian Amazon (Almeida et al. 1987). And the second one, regarded as being genetically more diverse, is in Ilhéus, at the State of Bahia. Together the two Brazilian collections comprise nearly 3,000 clonal and seminal accessions, showing a wide variability for most traits of genetic and agronomic importance as for production, resistance to diseases, gametic compatibility, pod and seed shapes, pod and seed sizes, plant architecture, flower characteristics, and so forth. Among those accessions are also the local selections of Bahia and Espírito Santo and the introductions

from other countries. One of the most intensive collections of wild cocoa in the diversity centre was done by the scientists of CEPLAC, covering various areas in the Brazilian Amazon alongside the tributaries of the Amazon River (Almeida et al. 1987).

While the collections of Trinidad, Costa Rica and Bahia, Brazil, have many genotypes resulting from breeding or planting populations, the one from Belém, Brazil, is characterised mainly by having more than 90% of its accessions from the wild.

In Africa, the larger and most representatives cocoa collections are the ones maintained by the 'Centre National de la Recherche Agronomique de Ivory Coast'(CNRA), and by the Cocoa Research Institute of Ghana (CRIG).

There is an international cocoa germplasm database, called ICGD, maintained by the University of Reading, in England, frequently updated and available on the Internet (www.icgd.reading.ac.uk) having phenotypic and molecular information for most of those accessions (End et al. 1992).

16.7 Breeding Objectives

The major objectives of most cocoa breeding programmes are productivity, seed quality and pest and disease resistance. More recently, emphasis has been on plant architecture, gametic compatibility and dwarfism.

16.7.1 Productivity

Whenever breeding cocoa for developing improved varieties is undertaken, the productivity is one of the main characteristics of any breeder concern. Planting materials should present high productivity so that they can bring compensatory returns to the producers. The production and its components should be appraised properly, because they are treated as polygenic characters and, consequently, highly influenced by environmental factors. The seed production per pod varies considerably within the same cocoa tree as between different trees. The number of ovules in the flower's ovary can determine the potential seed number, which is a character quite uniform. The pod index (number of fruits necessary to produce one pound or one kilogram of dried cocoa beans) is also used as a selection criterion, aiming to select productive varieties what facilitates the harvesting process. The larger the pod index, the smaller are the pods, the seed number or the seed size. Varieties producing over 1.5 kg of dry beans per plant are usually regarded as a good planting material. The productivity is also related to other traits, as plant size, gametic compatibility, flowering synchronism, plague resistance, plant management and the planting density.

16.7.2 Seed Quality

It is another important trait to be investigated in any cocoa breeding programme. Many characters, such as the physical-quantitative nature (seed size, seed shell, fat

content) or biochemical-qualitative (quality and hardness of the butter) are related to the quality of the cocoa seed. The seed size can be expressed by the seed index (average weight of the dried bean). Cocoa varieties with a large seed index are highly desirable. Usually the cocoa breeders try to select genotypes with seed index superior to 1.0 gram. Shell is an undesirable seed characteristic and presents almost no economic value. Seeds with reduced quantity of shell are preferred by the cocoa industries, because it is in the cotyledon that has the largest value in the seed. As the cocoa butter is the most valuable cotyledon component, it is of extreme importance to breed towards obtaining improved varieties with high fat content of good quality. The cocoa butter is compounded by several fatty acids and the fat hardness is related to the composition and proportion of these acids. Thus, cocoa varieties with higher fat hardness are also preferred by the chocolate industries.

16.7.3 Gametic Compatibility

The development of self-compatible varieties is desirable, since the effect of the gametic incompatibility on yield is quite complex and also indirectly concurs to provoke competition among plants in the population.

16.7.4 Dwarfism

Selection towards dwarfism is very important. Cocoa population with dwarf characteristic allows for easier plant management, harvesting and also the design of production systems in which the land can be used more properly. With such planting materials the establishment of plantings can be planned with higher density. Besides that, dwarf plants can also be explored as rootstocks since this genetic status has influence on the growth of the clones.

16.7.5 Plant Architecture for Easy Management

The cocoa breeders have interest in developing planting varieties with good architecture that make easier the management and the harvesting activities. These varieties can be used in different planting densities and allow the production of cocoa at a low cost. The angle of opening of the branches at *jorquete* level can define the final height of the plant. Plants with a low vigour may require less pruning.

16.7.6 Pest and Disease Resistance

Chemical control of pest and disease is expensive and also may represent an environmental hazard. The development of resistant varieties is economically desirable, environment friendly and also for increasing the efficacy of other less aggressive

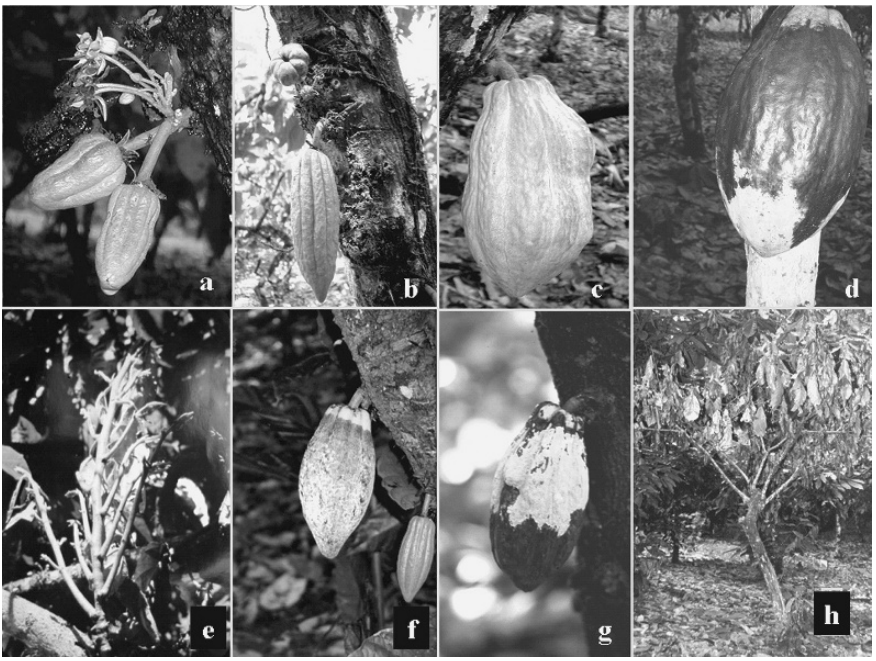

Fig. 16.4 Symptoms of some important cocoa diseases: Witches' broom (**a** and **b** – cushion broom and strawberry cherelles; **c** and **d** – diseased pod in field; **e** – vegetative broom); **f** – Black pod; **g** – Frosty pod rot and **h** – Ceratocystis wilt (*See* Color Insert)

control practices. Diseases such as, *Phytophthora* pod rot (or black pod), caused by *Phytophthora* species (*P. capsici, P. palmivora, P. citrophthora* and *P. megakarya*), witches' broom, caused by *Moniliophthora (ex-Crinipellis) perniciosa* (Aime and Phillips-Mora), vascular streak dieback (VSD), caused by *Oncobasidium theobromae*, frosty pod rot (Moniliasis), caused by *Moniliophthora roreri*, antrachnose, caused by *Colletotrichum gloeosporioides* Penz., Verticillium wilt, caused by *Verticillium dahliae*, and *Ceratocystis* wilt, caused by *Ceratocystis cacaofunesta* (Engelbrecht and Harrington, 2005), have been the main causes of pod losses and mortality of cocoa plants (Fig. 16.4). Most programmes put a lot of effort in breeding for disease resistance since in some regions the losses due to diseases can be as high as 100%. Cocoa pod borer (CPB) is an example of a serious insect pest in Malaysia and Indonesia. Development of varieties with desirable pod physical traits may reduce the pod losses by the CPB (Lamin and Sa'edi 1994).

16.8 Breeding Methods

16.8.1 General Breeding Programmes

The following flow chart (Fig. 16.5) summarises the majority of the cocoa breeding programmes.

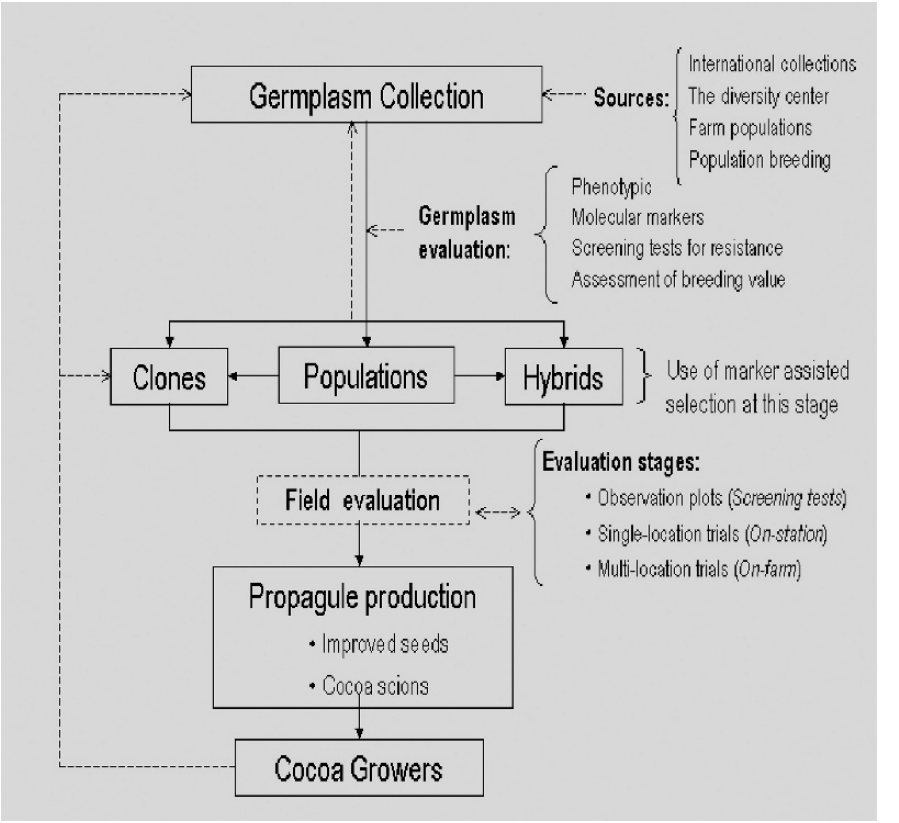

Fig. 16.5 General scheme of a cocoa breeding programme

16.8.1.1 Germplasm Collection

The first step for implementing a cocoa breeding programme is the introduction of germplasms. This can be done by making collections directly in the diversity centre and also in farm plantations or selecting or introducing elite varieties from other breeding programmes. Many expeditions have been made to the cocoa diversity centre aiming to collect germplasm. As these expeditions are quite costly, and usually selection of particular genotypes under those conditions is of low efficiency, the best way to collect wild cocoa germplasm would be by seeds. A sample of seeds from a specific region may represent more adequately the natural variability of the population. Besides that, by allowing these genotypes to recombine, randomly or not, the cost of maintaining the natural genetic variability of the species becomes less expensive, giving opportunities for important genes to express themselves.

Elite varieties that have been developed in other breeding programmes are also useful for the programme, even considering the risk of non-adaptation to regions different from those where they were selected. However, time can be gained using this strategy. In Brazil, for instance, such elite varieties introduced from other

countries have been widely used for breeding purposes. Another strategy to widen germplasms is selecting individuals on farmer's populations. This strategy has being used by cocoa breeders for some time now, with success. Examples of this are the Imperial College Selections (ICS) clones selected in Trinidad plantations, the United Fruit Selections (UF) clones selected in Costa Rica, the "Seleção do Instituto de Cacau" (SIC) and the "Seleção do Instituto Agronômico do Leste" (SIAL) clones selected in the Bahian populations. Many of these on-farm selections proved important for the Brazilian breeding programme, particularly in the development of hybrids.

16.8.1.2 Germplasm Preservation and Evaluation

Cocoa germplasm collections are usually preserved ex situ. The accessions are multiplied by vegetative propagation as clones. The propagation procedures usually adopted are budding or rooted cuttings. To avoid future problems with misidentification, especially when the budding procedure is adopted, rootstocks with an easily identifiable marker need to be used. One of such phenotypic markers is the whitish colour of the young leaves, as observed in the Catongo variety. In Brazil, in the collection of Belém, Pará, the cocoa accessions are preserved either as seedlings or as clones. Usually, in these collections, 10 plants represent each accession.

For some time, efforts were basically concentrated on the phenotypic characterisation of the accessions preserved in collections, and very little emphasis was given on the evaluation of traits of agronomic importance. This certainly was the case for the little success of the breeding programmes in developing superior improved varieties. The evaluation of agronomic traits in the collection is of fundamental importance for any breeding programme. Despite the limitations, such as: number of individuals per accession, non-randomisation of accessions, non-replication of the accessions in the field, age differences of the accessions, lack of common control varieties and others, the germplasm collection represents a great opportunity to preliminarily assess a large number of varieties, for many traits of agronomic importance, under the environmental conditions of the programme.

Some techniques have been suggested by Pires (2003) to overcome some of these limitations, thus increasing the reliability of the information obtained from cocoa germplasm collections. Data calibration can be used to overtake differences in vigour or age. The yield performance and some measures of disease resistance can be adjusted by the trunk diameter. In Brazil, the cocoa collection of Belém is being transferred to Altamira, Pará, and the accessions are being randomly replicated in small blocks with common control varieties. The same is in progress with the new accessions introduced in CEPLAC, Bahia. With this procedure, it will be possible to estimate the experimental error.

16.8.2 Population Breeding

Making crosses in cocoa is an easy process, because the flowering season extends for a prolonged period, thus facilitating, pollinations. The only way of accumulating

favourable alleles in a single variety is by inter-crossing recurrently individuals with desirable traits. Even though, recurrent selection has been ignored in cocoa for many years some work was carried out in this area during 1940s in Trinidad.

Most of the cocoa programmes do not go further than the first generation of breeding, in which clonal accessions from the germplasm collection are inter-crossed and the best hybrids or full-sibs families recommended for planting. And, for the following generations of crossings, no selection work is done for identifying the parents of the new hybrid progenies. The limitation in information on the accessions present in the collections generally concurs to the excessive number of unnecessary crossings and, often with very little gain. Equally, no recurrent breeding was done to support cloning and hybrid programmes. Usually clones were selected either on farmers' populations or within progenies and then recommended for planting, after being tested.

In cocoa some strategies have been adopted, one in which crosses are made without following a rigid mating design, called here 'unstructured strategy', and in the other in which crosses are made according to a mating design, such as factorial, diallel or other, called here 'structured strategy'. More recently, Brazil adopted these two breeding strategies due to an increasing demand for resistant clones after the presence of witches' broom in the main producer region of the country.

With the unstructured strategy the breeders take the advantages of the infrastructure (progenies, germplasm collection, farm populations, etc.) and information available to breed populations giving support especially to a clone programme. Some complex crosses are produced aimed to achieve major breeding objectives. Usually, several parents having characteristics of interest are selected and pairs are formed trying to complement characteristics from two clones (e.g., resistance from a clone, with high yield and seed size from another clone). The population size depends on the level of heterozygosity of the parents involved and the interest on the progeny. As the evaluations are done by plant individually, it is necessary that an adequate plant management be practiced to avoid the competition effects. The main advantage of this strategy is the involvement of several parents, with fewer crosses, and these crosses are directed for the desired ideotype. Furthermore, it is possible to save space and time in evaluation by planting the cocoa trees in higher density and practicing early selection. However, it has the disadvantage of not offering information on the breeding value of the parents, which would otherwise increase the genetic gain.

In the structured strategy, a mating design is planned involving parents with traits of interest. This strategy concentrates efforts in fewer parents, by combining them according to an adequate mating design that allows the estimation of the breeding values and other genetic parameters useful to the programme. For example, in Brazil three factorial mating designs were made comprising of 64 crosses each aiming to combine: yield, fat quality, resistance to witches' broom, self-compatibility and other important traits (Pires et al. 1999). Besides that, sources of resistance to witches' broom, potentially carrying different genes of resistance were crossed aiming to increase durability of the resistance to this disease. And, with the aid of molecular markers, diversity among parents was maximised, thus increasing the

Populations		R				P			
	Parents	1	2	3	4	5	6	7	8
R	9	x	x	x	x	x	x	x	x
	10	x	x	RR	x	x	x	RP	x
	11	x	x	x	x	x	x	x	x
	12	x	x	x	x	x	x	x	x
P	13	x	x	x	x	x	x	x	x
	14	x	x	PR	x	x	x	PP	x
	15	x	x	x	x	x	x	x	x
	16	x	x	x	x	x	x	x	x

R – Resistant to witches' broom
P -- Productive

Fig. 16.6 Factorial-mating design for breeding base populations

chances of accumulating different favourable alleles from different sources. This strategy was set up under a system of reciprocal recurrent selection (Fig. 16.6). Out of the eight witches' broom resistant parents, four were used as male (parents 1–4) and four as female (parents 9–12). The same was done with the high yield and quality parents. This way, the crossings between the parents 1 to 4 with 9 to 12, will recurrently accumulate alleles of resistance and those between the parents 5 to 8 with 13 to 16, will accumulate alleles of high yield and quality. The crosses between the parents 1 to 4 with 5 to 8 or between 9 to 12 with 13 to 16 will not only allow the assessment of the specific combining ability, but also the selection of individuals combining the resistance and yield and quality.

The selection efficiency can be increased in these two strategies by replicating every individual plant, what will reduce the environmental effect during the selection process.

In Trinidad, for example, the unstructured strategy was also used. Several crosses were produced with the involvement of the clones Sca-6 and Sca-12 (resistant to witches' broom, but with small seeds) and other clones with high yield and large seed size (e.g., ICS-1) and/or high resistance to *Ceratocystis* wilt (e.g., IMC-67). This programme resulted in the selection of the series TSH and TSA, widely used in most breeding programmes.

16.8.3 Derivation of Cones and Hybrids

Whenever breeding cocoa populations is undertaken it always opens opportunities for selecting clones and hybrids. Depending on the emphasis of the programme, the option can be made for parents with higher or lower level of heterozygosity. Evidently, if the emphasis is given for selection of clones, crosses involving parents with higher level of heterozygosity would be more interesting, because the recombination would facilitate, to a greater extent, individual plant selection within and between families. However, if the interest were for hybrids, parents with a higher level of homozygosity, because it would produce more uniform families and in this case selection should be restricted basically to among families. The quality of the information from the accessions in the germplasm collection is a decisive factor in the success of development of hybrids and clones.

16.8.4 Testing and Selecting Clones and Hybrids

During the development of clones or hybrids, standard procedures for field evaluation need to be followed in order to increase the efficiency of the varietal selection. A brief description on these evaluation procedures is given as follows:

16.8.4.1 Clones

The main sources of clones to be tested are: elite clones introduced from other countries, on-farm selections, and on-station selections made in the progenies developed in the recurrent selection programme. It is necessary to produce clonal gardens to attend the propagules demand for the establishment of clone trials. This can be done by grafting or budding on basal chupons of adult cocoa trees, to speed up the process of obtaining propagules. It is advisable that the clones be tested in three steps as shown in Fig. 16.5. In the first step, a large number of clones will be assessed and screened on-station for disease and pest resistance, seed quality, early seed germination and other important traits for three to four years in observation plots. A less intensive selection will be practiced when choosing the clones for the second stage of evaluation. At this stage, clones are tested in on-station clonal trial, following appropriate statistical design, with replicates. If self-incompatible clones are present, the field design needs to assure good husbandry regime. A complete randomised clonal trial will guarantee such a regime and avoid the masking effect on yield performance of these clones.

In the third and final step, the most promising selected clones are tested in multilocation on-farm trials, with statistical design not much different from the previous one. The choice of locations needs to be carefully studied to allow for the environments to be sampled appropriately, using as a base important factors such as, soil, climate, fungal population and others. In these clone trials, two to four common clones should be included to serve as controls. The use of more uniform rootstocks certainly will reduce the effect of rootstock x clone interaction, but the use of

cuttings for the establishment of the clone trial eliminates this effect completely, leading to more reliable selection. The grafting of clones on basal chupons of adult cocoa trees accelerates the growth, during the first two years. However, it leads to a tremendous variability within clones. This may be explained by the fact that the chupons do not develop uniform root systems and also by the strong competition between the clone and other chupons that grows especially after the trunk of adult cocoa has been removed close to the soil. The management in these trials should be as close as possible the one adopted by the farmers.

16.8.4.2 Hybrids

When building populations, families can also be selected for testing as hybrids. The hybrid evaluation follows almost the same steps as clones. At the first stage families can be evaluated either in observation plots of at least 60 plants per family over a period of 5–8 years. During this period, in every family, individual plants are evaluated for many important traits, such as yield, behaviour and resistance to diseases and pests. The families with better field performance and lower variability are selected for the second stage of evaluation. At this stage, information on the genetic value of the parents will be available. This can also be used to produce combinations by crossing parents with high general combining ability. Evidently, as genetic parents chosen were initially distant, for starting the breeding programme, hybrid vigour is expected to express more frequently. In the next stage, the combinations are tested in on-station hybrid trials for a period of 6–10 years, according to an adequate statistical design, with three to four replications and plots with at least 12 plants, plus control varieties. The best performing hybrids are selected for the stage 3 of evaluation. They are tested in multi-location on-farm trials for approximately 8–10 years, using an adequate statistical design, with 4–5 replications, and 10–16 plants per plot. Ideal plant management needs to be practiced for the families to express the true productive potential in distinct environments. Field data collection at the stages 2 and 3 is done on a plot basis. Sometimes, very good hybrid combinations identified in stage 1 can be tested directly in stage 3 and this is also common in clones. Within a participatory approach, clones or hybrids can also be evaluated or validated by establishing small on-farm observation plots over a range of environments. This procedure allows the selection of clone or hybrid varieties in a shorter period of time, with also reasonably good precision.

16.8.5 Strategies for the Production and Distribution of Popagules

16.8.5.1 Improved Seeds

Improved seeds are produced in fields duly planned to facilitate pollinations. To produce hybrid seeds, the two parents are planted side by side and the flower buds that are going to receive the pollen grains need to be protected one day before anthesis. The hand pollinations are done with the aid of a pair of forceps. The

stamens are removed from the flowers and the anthers are blushed on the style and stigma of female flowers. Alcohol is frequently utilised for the sterilszation of forceps and to prevent against pollen contamination. Although these areas could be programmed by taking advantage of the self-incompatibility of some parents, the self-pollination always occurs because of the mixture of pollen that inhibit the incompatibility reaction leading to seed contamination by the production of cross- and self-pollinated seeds. A well-trained man can do about 400 hand pollinations per day, but by protecting the flower cushions before the flowers open, this number is reduced to about 250, as greater time is involved, but with the advantage of better pollination quality. The flowers are generally collected in the morning period and then conditioned inside a styrofoam box to preserve the pollen in a good state for pollinations.

16.8.5.2 Cocoa Scions

To guarantee the supply of cocoa scions for the producers, clonal gardens duly dimensioned are organised at farm level or in central units, a kind of Bio-factory or *Biofabrica*, as in Brazil. Usually released clones are initially multiplied by vegetative propagation, through grafting. This procedure can be done on lateral or basal chupons of adult cocoa trees. A sufficient supply of propagule will be obtained over a period of a year. The cocoa scion can be used for the purpose of grafting or for producing rooted cuttings.

Trinidad and Costa Rica developed the technology of rooted cuttings to propagate their local clone selections and, later on, it was adapted by other producer countries, as an example; by big companies in Ecuador, that are using this procedure to multiply CCN-51 clone in large scale. In Malaysia, they adopted the budding on young seedlings (green budding), as well as the grafting directly on the trunk. In Brazil, two propagation systems have been adopted more frequently: rooted cuttings and grafting on basal suckers or *chupons*. The rooted cuttings technology used in Brazil are quite different from that developed in Costa Rica and Trinidad, being an adaptation of the technology used by large eucalyptus enterprises for mass production of rooted cuttings. The cocoa scions are rooted inside *tubetes* following a defined protocol. The large-scale production of rooted cuttings is being done by the "Instituto Biofabrica de Cacau", in Ilhéus, Bahia, Brazil.

The grafting technology is the most widely propagation process used in Brazil by the producers and underwent considerable improvement. The grafting on basal chupons of adult cocoa trees was a strategy adopted to reduce the cost of the substitution of the decadent susceptible plantings by resistant clonal varieties.

16.8.5.3 Production and Distribution of Hybrid Seeds

The fields for the production of hybrid seeds have been directed at taking advantage of the self-incompatibility condition of the female parents in some countries. This procedure was adopted strategically aiming to reduce the cost with hand pollinations. Thus, by planting self-compatible and self-incompatible parental clones

in alternate lines, the collection of the seeds was done solely in rows of the self-incompatible parents, because it was expected that the hybrid seeds could be produced as a result of the cross-pollination. Later, it was discovered that mixtures of compatible and incompatible pollen could also inhibit the reaction of incompatibility in the self-incompatible flowers, allowing also the production of self-fertilised seeds in the same pod. This contamination might have happened quite frequently and, certainly, contributed significantly in increasing the heterogeneity within population and, consequently reduction of bean production. This was one of the major problems that were experienced by those countries that used mixture of hybrid seeds as planting material, especially Brazil, Costa Rica, Ivory Coast, Malaysia, and other countries.

CEPLAC released to cocoa producers a mixture of improved hybrid varieties composed of pods from several superior hybrids selected in experiments. In Bahia, this mixture was composed by more than 30 hybrid varieties. The option for the mixture was based on the concept of the genetic homeostasis, since the heterogeneity in the population would bring the advantages of increasing the stability across environments and reduction in pod losses due to diseases, as for instance: *Phytophthora* pod rot. However, this high heterogeneity had the disadvantage of a drop in yield, more competition between plants with a consequent increase in mortality of cocoa trees and reduction in the stand of plants. Further, many hybrid combinations had as parent a self-incompatible clone, which resulted in progenies with segregation of plants for self- and cross-incompatibility. The interactions of all these factors have a great importance in the general development of the planting variety. In general, self-incompatible cocoa trees grow more vigorously than self-compatible ones. And in situations where the cocoa trees were not pruned properly to give an adequate canopy formation, the self-compatible plants became dominated and, consequently, the negative effect of the self- and cross-incompatibility expressed more drastically and reflected in low productivity. This fact became more accentuated in situations wherein many producers selected from the pod mixture of superior hybrids; pods that presented the traits they like most. While choosing pods with good appearance for planting, farmers ignored the fact of concentrating on the frequency of self-incompatible genotypes in the population, being unaware to the consequences on productivity. The great majority of the hybrid plantings had good productions during the fifth to eighth years of age, after then initiated a continuous drop in production, also followed by a reduction in the stand of plants. Conscious of this problem, CEPLAC started to restrict the composition of the mixture to fewer hybrid combinations involving only self-compatible parents, with larger uniformity for field performance, plant architecture, production, disease resistance and for other important characteristics of fruit and seeds. The new composition was more homogeneous and was released to producers after 1983. This resulted in more uniform and productive populations. The best hybrids developed by CEPLAC produced under experimental conditions averaged about 2000 kg of dry seeds per hectare. Although productivities superior to 1500 kg of dry seeds per hectare for the hybrid mixture at farms level was not reached in most of the farms.

16.9 Marker-Assisted Cocoa Breeding

Until recently only a few markers were available to cocoa breeders, particularly phenotypic markers (axil spot, leaf and cotyledon colors) and isozymes (Lanaud 1986; Yamada and Lopes 1999; Yamada and Guries 2000). However, the use of these markers has severe limitations in breeding, particularly because of their low level of polymorphism per locus and the number of loci available. With the development of molecular markers based on DNA these problems were overcome. These DNA markers have the advantage of covering the whole genome, being particularly adequate for diversity studies, mislabelling analyses and mapping of genes of interest.

The use of molecular markers for annual crops like tomato, maize, bean, has had a tremendous impact on breeding for these species. In perennial crops like cocoa, the impact of this technology in the development of new improved varieties should be even greater, considering its perennial nature in obtaining and assessing generations and the need for early decisions making (in selection, elimination of duplicates in germplasm collections, elimination of similar individuals in breeding populations).

In cocoa, the molecular makers can be used with multiple objectives, such as

Identification of mislabelled plants in germplasm collections;
Understanding of the level and shape of the diversity in breeding populations;
Establishing base populations;
Estimating heterozygosis to identify inbred clones;
Mapping genes of agronomic interest; and
Marker assisted selection (MAS).

The CFC/ICCO/BIOMOL project on *The use of molecular biology techniques in a search for resistant varieties to witches' broom disease of cocoa* made an important contribution in all these six objectives, by using molecular markers as tools in operational breeding programmes in Brazil, Peru and Ecuador.

16.9.1 Mislabelling in Germplasm Collections

Cocoa germplasm collections are usually maintained ex situ, with a high number of accessions. As the cost for their maintenance and labelling is quite high, the problems of mislabelling are also quite frequent. Besides that, interchange of germplasms in cocoa is very common and some countries changed the original name of the accessions, increasing furthermore the resultant disorder. The mislabelling in international cocoa germplasm collections can be as high as 20–30% of trees in collections (Christopher et al. 1999; Motilal et al. 2002; Saunders et al. 2001).

Efforts to correct mislabelling in cocoa collections are well underway, a great part undertaken by in the US Department of Agriculture collections (Saunders et al. 2004). This initiative aims to fingerprint all cocoa accessions in the major collections in Latin America, using microsatellite markers. However, in a smaller scale,

some breeding programmes have taken advantage of molecular markers to clarify mislabelling in their own collections (Faleiro et al. 2002).

16.9.2 Diversity in Breeding Populations

The knowledge in the extent and nature of the variability in breeding populations is important to avoid closely related parents. This saves resources and maximises diversity. In cocoa, few genes have been mapped and only recently research has been undertaken aimed at identifying genes that control traits, particularly disease resistance. Therefore, this knowledge at the moment is insufficient to assess the diversity for agronomic traits. However, it can be assumed that diversity in the genome using a large number of markers is correlated with the variability in the genes in general, especially those of agronomic importance. Thus, with the lack of direct information on diversity of genes of agronomic interest, molecular markers become an important tool to estimate that diversity. Diversity in cocoa has been characterised in wild germplasms collected from the centre of origin (Mota 2003), in germplasm accessions in general (Marita 1998; Faleiro et al. 2004a), in specific populations like that in *Criollos* (Motamayor et al. 1997) and in farmer selections (Faleiro et al. 2004b,c). Overall, the Upper Amazon *Forasteros* have shown the largest genetic diversity (Pires 2003).

16.9.3 Establishing Base Populations

The knowledge of genetic variability is essential for breeding populations. By eliminating closely related parents the resources allocated to evaluate individuals, genetically similar, can be saved. Also, the diversity in the breeding population is desirable to increase the chances of genetic gain for the target traits. As mentioned, diversity based on molecular makers, in the absence of information on diversity at the gene level, can be a useful tool to identify individuals to be included in base populations.

The cocoa breeders in the Brazilian programme have used this strategy. Pires et al. (1999) aiming to form several base populations pre-selected clones in the germplasm collection based on phenotypic traits of interest, including yield, witches' broom resistance and butter quality. Those pre-selected individuals, then, were fingerprinted with molecular markers to assess the genetic distance among them. Since the idea was to include a set of eight clones with resistance to witches' broom and a same number of other clones with the other traits, those eight clones of each group with maximum diversity were selected to be included in the base population. This set of 16 clones was inter-crossed in a factorial mating design as shown in Fig. 16.6. In this strategy, the eight clones resistant to witches' broom, with a high genetic diversity and, hopefully, with different genes of resistance are inter-crossed among themselves (RR, in Fig. 16.6). The same occurs with the eight clones with high production (PP). At the same time, the resistant clones are crossed

with the high yielders. The selection of parents for the next cycle of breeding follows a scheme of reciprocal recurrent selection. Up to now three populations formed as in this factorial design with 16 clones each and two or three replications are planted in the field.

16.9.4 Heterozygosis of Parents

Cocoa breeders worldwide have undertaken interclonal hybridisation to improve varieties, since the discovery of heterosis in cocoa in Trinidad (Russel 1952). However, due to the allogamous condition of the cocoa species, most of clones used as parents present a high level of heterozygosis, yielding hybrids with a high variability.

In an attempt to reduce that variability in the hybrid population, some breeding programmes started developing inbred parents to increase the level of homozygosity. In Trinidad, for example, elite clones (ICS-1 and ICS-45) were self-pollinated by K.S. Dodds in 1947 and the S_1 lines crossed (Bartley 1953). In Brazil, clones of the series SIAL and SIC, selected from the local common population (Carletto et al. 1977) were widely used for producing hybrids. Later, generations of selfing were advanced in some of these SIC and SIAL clones, and in other clones, in Brazil. With the emphasis on the development of clones, after the witches' broom outbreak, that programme was discontinued.

Since in each generation of self-pollination, half of the loci will be in homozygosis on an average; some individuals may have the level of homozygosis higher than 50%. If these individuals are identified, the process of inbreeding is much faster, making it feasible to obtain inbred parents. Molecular markers can be very useful in this task. The level of homozygosis has been determined for many clones (Mota 2003; Yamada et al. 2003; Faleiro et al. 2004b,c) and, in CEPLAC, progenies have already been produced considering this information.

16.9.5 Mapping Genes of Agronomic Interest

Positioning genes of interest or quantitative trait loci (QTL) in genetic maps constructed with molecular markers has called the attention of breeders of many plant and animal species. The main reason is that knowing the position of the gene in this type of map automatically markers close to it are known that tends to be inherited together. So, the presence of these markers in individuals is an indication of the likely presence of the gene. Many linkage maps have been developed for cocoa aiming to associate molecular markers to agronomic traits. The major traits mapped are resistance to *Phytophthora* pod rot caused by *Phytophthora* spp (Crouzillat et al. 2000b; Risterucci et al. 2003), yield (Crouzillat et al. 2000a) and witches' broom resistance caused by *Moniliophthora perniciosa* (Queiroz et al. 2003).

16.9.6 Marker-Assisted Selection (MAS)

The construction of linkage maps allows the association of markers to agronomical important traits and subsequently marker-assisted selection (Tanksley et al. 1988) speeding up the breeding process. This is a type of indirect selection, in which selection is done on the marker to have a response on the trait of interest. As the selection is based on DNA information (markers), this selection can be undertaken as early as DNA can be extracted from the plant, speeding up the breeding cycles. Again, as selection is done at the DNA level, the trait does not need to be expressed before selection is practiced. So, when selecting for disease resistance, for example, the potential selections do not need to be exposed to the disease to allow the identification of those carrying genes of resistance. Therefore, making it possible to initiate preventive breeding against diseases while absent in a region or country.

Another advantage of marker-assisted selection is the accumulation of genes whose effect cannot be perceived based on the phenotype. For example, when breeding for disease resistance it is very desirable to accumulate (pyramiding) genes of resistance, in order to reduce the chances of pathogen breakdown to existing resistance. However, sometimes individuals having alleles of resistance in a single locus are immune or highly resistant. When two individuals are crossed having alleles of resistance in different loci, the level of resistance in individuals accumulating alleles of resistance in the two loci may not be different from that of individuals having such alleles in a single locus. However, theoretically, that individual having alleles of resistance in both loci will be less subject to having its resistance overcome. Using markers associated to alleles of resistance at each loci, allows them to be tracked in the breeding population, and so the identification of individuals accumulating alleles of resistance. This can be done early in a breeding programme even for diseases not present in the region. This is particularly important in tropical perennial crops, like cocoa, which is attacked by a host of severe diseases, with repeated cycles of disease infection and selections in the pathogen population.

16.10 A Synthesis of the Major Cocoa Breeding Programmes

16.10.1 American Continents and Caribbean

16.10.1.1 The Brazilian Programme

Historically the first introduction of cocoa into the State of Bahia was in 1746. Seeds from the Lower Amazon population were planted at the Southeast of this State. Local farmers established new plantings over about two centuries, using the seeds derived from this original planting. During this period they improved the population by mass selection that gave rise to four basic varieties, that is, var. *Comum*, var. *Pará*, var. *Parazinho* and var. *Maranhão*.

In 1940s, the Bahia Cocoa Institute (ICB) initiated a programme of selection of individual plants within the local population. The on-farm cocoa tree selections were

based mainly on the evaluation of yield and its components, over a certain number of years. This resulted in the selection of interesting genotypes as the ones of the series SIC. Further, during this period, some white seed mutants were identified, as, for example, the *Catongo* selections. In the following decade, this work continued with the selections made by the Eastern Agronomic Institute and the Experimental Station of Goitacazes giving origin to the SIAL and EEG selections, respectively. These local selections were reasonably productive, but many of them did not present resistance to pests and diseases, especially *Phytophthora* pod rot. In addition, they showed very low variability for most important traits.

CEPLAC was created with the objective of rehabilitating the cocoa farms in Bahia, and with the obligation of providing cocoa growers superior varieties compared to existing ones. CEPLAC started to introduce from other producing countries, especially Trinidad and Costa Rica, including various important clonal accessions resistant to pests and diseases, to be used directly as cultivars or to be used in the local breeding programme. At the same time, an ambitious breeding programme was installed at CEPLAC, giving more emphasis to the development of hybrid varieties. A total of approximately 600 full-sib progenies were produced by undertaking crossing of the following types: Local selections × *Trinitarios*; Local selections × Upper or Lower Amazons; Local selections × *Criollos; Trinitarios* × *Trinitarios, Criollos* × *Trinitarios*, Local selections × Local and Amazon × Amazon. The progenies were tested locally and over different environments in progeny trials with appropriate statistic and genetic designs, enabling the assessment of genetic values of the progenies and also the clones as parents. The traits normally evaluated were seed production, seed and fruit indexes, percentage of seed shell, the incidence of pod diseases and other important characters.

The large number of crosses produced is a reflection of the little information then available on the germplasm collection, especially on the clones used as parents. A general analyses allowed grouping of these crosses, and showed that the hybrid combinations involving the local selections and the clones Sca-6 or Sca-12 were ranked as the most productive, but with very small seeds, that is, inferior to 1.0 g, the minimum value stipulated by the chocolate industry as acceptable. The hybrid combinations among the local selections were, in general, superior to the ones with *Trinitario* clones in terms of production. Many crosses with clones introduced from Costa Rica, especially the ones with UF-613, presented lower incidence of *Phytophthora spp* in fruits and productivities similar to the combinations among the local selections. When the local selections were crossed with these clones the result was slightly superior yield performance, but with larger incidence of black pod. The hybrids with the Upper Amazon clones (Sca-6, Sca-12 and IMC-67) also presented larger precocity in production, but a decline of production was observed from the 6th year onwards, and this may be due to the effects of competition among plants.

With the expansion of the activities of CEPLAC in the North of Brazil, a parallel programme of variety improvement was also carried out aiming to develop superior hybrids. Great emphasis was given to the selection of progenies with resistance to the witches' broom, an endemic cocoa disease of the Amazon region. The results showed combinations between the Amazon selections made by CEPLAC with the

introduced clones as P-7, Ma-15, IMC-67, Sca-6, Sca-12 and others, were more productive. Again, the combinations with Sca-6 stood out as being with the lowest levels of infection to witches' broom, while the ones with the Bahian selections behaved as highly susceptible. Although in Bahia, the hybrids have been improved for productivity, disease resistance and quality characters almost no preventive breeding for exotic diseases was contemplated, as for the case of witches' broom and *Ceratocystis* wilt, caused by the fungi *Moniliophthora perniciosa* and *Ceratocystis cacaofunesta*, respectively. Even considering the geographical and temporal isolation from endemic sources of witches' broom, it appeared in Bahia in 1989, causing a devastating impact on the local socio-economy well-being of the rural communities and its reflection in urban areas (Pereira 1998, 2000a,b; Pereira et al. 1990, 1996; Pereira and Valle 2002). The cause of the rapid and explosive spread was due to the susceptibility of, by far, most of the varieties in the region and erosion in time, of any resistance that may have been present, further, climatic conditions were ideal for disease development.

Since a disease management strategy, in all its components of control were proved to be inadequate over the 100 years existence of the disease, CEPLAC had to adapt a programme of genetic improvement in support, directed at release of resistant varieties, in the region of Bahia, which had its virtually totally dependence on a mono-crop, cocoa. Clonal selection was regarded as the most effective strategy for developing improved cultivars. Thus, to support the clonal selection programme, breeding populations were produced aiming at the pyramiding of genes for resistance to witches' broom mainly, with the enlargement of the genetic base of resistance and, consequently, increasing the field resistance of the plants. When building these populations the information available on genetic distance of the parents was taken into account, measured by DNA markers. The possibility of association of characters of genetic and agronomic importance, such as resistance to other diseases, high production, large seed size, large number of seeds per pod, thickness of pod husk, self-compatibility, seed quality, and so forth, was also observed. After the presence of witches' broom in Bahia several populations were bred at CEPLAC that gave rise to more than 300 selected productive and resistant genotypes. Besides, farm populations under high witches' boom disease pressure, clearly provided an ideal selective environment for determining resistant planting materials. Many resistant mother trees were identified, evaluated for a period of one to two years and then selected and classified as VB selections, a temporary acronym that stand for *Vassoura-de-Bruxa* (= witches' broom). Later on, the methodology and criteria for selecting these planting materials were also passed to the local producers and the extension agents of CEPLAC. This gave the start to the participatory process that has resulted in a pre-selection of over five thousands resistant and productive mother trees; some of them have been exchanged among farmers and widely planted.

More recently, the CFC/ICCO/IPGRI Project on *Cocoa Productivity and Quality Improvement, a Participatory Approaches* supported technically and financially participatory selection for the search of planting materials in ten producing countries, including Brazil. Inoculation screening tests for resistance to *Phytophthora* sp, *Moniliophthora perniciosa* and *Ceratocystis cacaofunesta* have been done for

evaluating the genetic value of these selections. Many of them are being tested in several multi-location clone trials with the participation of farmers.

The Latin American cocoa producers have to learn how to live with the witches' broom as an endemic disease, maintaining in the forefront renewal of selected resistant varieties on their farms. The control of disease has been facilitated with the epidemiological studies and understanding the biology of *Moniliophthora* and the pathogen × host interaction. Further, with some level of resistance of the varieties, other components of integrated management (phytosanitory, chemical and biological control) in combination, further strengthen management strategy, while reducing the use of costly inputs and thus allowing for economic disease management.

The studies with molecular markers have shown the existence of genetic variability in the population of *Moniliophthora perniciosa*. And also, co-evolution of the fungus on the populations formed with those selected resistant cocoa trees. Even a classification that allows for determining the biological specialised forms of the *Moniliophthora* (physiologic races) is needed. This is of fundamental importance to breeding for the development of resistant varieties to the witches' broom. The selection progress depends on the knowledge gained on differentiation of the host × pathogen interaction. Disease resistance of a variety depends on both genotypes of the host (cocoa tree) and the pathogen (fungus), following expose of the host to the appropriate physiologic races of the pathogen (Figs. 16.7 and 16.8).

Fig. 16.7 Examples of cocoa clones selected and under testing in CEPLAC (*See* Color Insert)

Fig. 16.8 Farm selections used as planting material in Bahia, Brazil (*See* Color Insert)

The field evaluation is the most efficient manner for selecting resistant cocoa varieties by exposing them to the pathogen population. But this kind of evaluation is quite demanding in resources and in time and the validity of the genotype responses is limited to those environments where the varieties were tested.

Other methods that are frequently used for early selection of resistant cocoa trees are the artificial inoculations. They have been used in the Cacao Research Center (CEPLAC/CEPEC) for screening genotypes for resistance to *Phytophthora* spp, *Ceratocystis cacaofunesta, Verticillium* sp and *Moniliophthora perniciosa*. Young seedlings or clones are uniformly inoculated through a semi-automated conveyer-belt system with a suspension of spores of *Moniliophthora perniciosa*, of known concentration. After 30 days the genotype reaction is observed for the occurrence of symptoms of the disease. This system works very well for seedlings, but needs to be improved for clones, due to the difficulty to synchronise with the presence of uniform of flushes of young leaves to inoculation at determined test periods. The inoculation on cuttings seems to be quite promising a process due to the fact that there is no interference of the rootstock on the clone, but it needs further improvement.

Currently, most of the clone varieties available to the cocoa producers in Brazil are self-incompatible. Pollination tests need to be done to verify the cross-compatibility and also the level at which it occurs. In adopting a polyclonal planting system, it is necessary to use cross-compatible clones that present a high frequency of fertile pollinations. Otherwise, the effects of gametic incompatibility on the production will be much accentuated that can be noticed through the lack

of uniformity in pod size, number of seeds per pod and fewer pods per plant. The inclusion of self-compatible clones certainly reduces the negative effects of the cross-incompatibility. Besides, the size of those clones should be observed when making any clone composition, because the use of clones with different sizes will make it difficult to handle and consequently will induce the competition among them.

In addition, most clones that are being recommended for planting do not present any resistance to *Ceratocystis cacaofunesta*, usually disseminated through wounds caused on cocoa trees by cutting or tilling tools. Therefore, to control spread of the disease care is necessary in removal previously attacked plants from the area and the need for constant sterilisation of the pruning and cutting tools. The control of this disease can also be done through the use resistant rootstocks, but the availability of these is quite limited. For that reason, the use of rooted cuttings has to be avoided as the whole plant may succumb to infection, facilitating disease spread during the application of cultural practices such as pruning, de-suckering, pod harvesting and weeding. Therefore, in the absence of information on resistance, monoclonal planting systems are not recommended. The inoculation screening tests have been used for selecting resistant clones to *Ceratocystis cacaofunesta*. The results of the tests were not motivating, indicating that resistance is quite rare among the tested genotypes and that the Bahia isolates are more aggressive compared to ones from Ecuador. The inoculation technique needs to be improved for the better differentiation of resistant genotypes.

Since 1960s there was a large demand for genetically improved cocoa seeds for the implementation of the PROCACAU, a programme aimed at the renovation and expansion of the cocoa cultivation, and for this CEPLAC distributed over 100 million seeds per year. It began with the establishment of hybrid seed production sites parallel to the installation of on-station and multi-location variety trials. Later, CEPLAC expanded its activities to the North of Brazil and in 1976, implanted other on-station sites for the production of hybrid seeds in Altamira, Manaus and Ouro Preto d'Oeste in the States of Pará, Amazon and Rondonia, respectively. Besides, these introduced selections that were used as parental clones in Bahia, other local selections were made by CEPLAC in Bahia and Amazon regions. Once the most promising and well-adapted combinations were identified in the local hybrid trials these sites had to be re-structured for the production of new varieties. In 1986, CEPLAC distributed in the Amazon around 15 millions hybrid seeds involving mainly the clones Sca-6, P-7, Ma-15, Ca-6, IMC-67, SIC, ICS-1, Ma-11, Pa-150 and Be-10. Currently CEPLAC is officially recommending the planting of 24 clones that need to follow a planting plan, as most of them are self-incompatibles. The farmers are also encouraged to use some of the farm selections, especially the ones that stood the test of high field inoculum pressure of *C. perniciosa* and presented good field performance in various farms. Monoclonal plantings are not being recommended because they increase the risks of dissemination of disease like *Ceratocystis* wilt and also the chances for the evolution of pathogens in the resistant population.

Just after the presence of witches' broom disease in Bahia, in 1989, there was a great demand for resistant varieties. The varieties that were recommended by

CEPLAC until then, did not present any resistance. The first resistant hybrid variety to be released at that time was variety *Theobahia*, in 1995. It is a progeny between Sca-6 and ICS-1 that produces in average 2160 kg of dry seeds per hectare. This progeny was originally released with the purpose of forming small populations in a large number of farms and, later on, to serve as support to the growers to make their own selections and so substitute susceptible plants in their population for those selected as resistant in the same family, through grafting, improving thus the level of resistance of the population. These selections could also be used as planting material for the establishment of new plantings. Later, two other resistant hybrid varieties produced (Sca-6 × ICS-6 and Sca-6 × ICS-8) were released and were named *Theobahia* 1 and 2, respectively. These varieties had to be withdrawn because of the narrow base of resistance to witches' broom and the susceptibility to *Ceratocystis* wilt.

The Ecuadorian Programme – Between 1880 and 1890, Ecuador was the world's largest cocoa exporter. The country has an important cocoa variety, characterised for its attractive organoleptic qualities and recognised worldwide for its floral aroma and yielding the finest chocolate with the peculiar *arriba* flavour, derived from *Cacao Nacional* (Enriquez et al. 1994), investigated by the *Instituto Nacional Autónomo de Investigaciónes Agropecuarias* (INIAP). In the past, although the local breeders were quite concerned in preserving the traditional flavour of the *Cacao Nacional*, the cocoa breeding in Ecuador were strongly influenced by the Trinidad's programme. During the cocoa boom, Ecuador came to produce around 80,000 MT/year in 1916 (Flower and López 1949). During this period, Trinitario selections were also introduced as planting materials. But the presence of cocoa diseases, such as frosty pod rot and witches' broom caused enormous reduction in production to the level of 10,000 MT/year by 1930 (Arosemena 1991). To this economic, social and technical disaster was added the effects of lack of transport, decreased demand in the international markets during the First World War and the economic depression of those years. Many farms were abandoned and sold as small and medium holdings. By the end of 1930s the plantings were renewed with the seeds collected from trees tolerant to diseases, giving origin to the current variety complex, denominated national × Venezuelan hybrid. This hybrid is the result of the natural crossing between the survivor trees of the national variety and the Trinitario variety, introduced from Venezuela at the beginning of the century.

The first attempt to overtake this problem came with the gathering of germplasm resistant to witches' broom in 1937 in the Amazon (Pound 1938). This resulted in the selection of Scavinas and IMC's amongst others. With these, a breeding programme was initiated with the object of developing superior hybrids with resistance to witches' broom. Crosses were planned in an attempt to promote the introgression of witches' broom resistance genes into the *Cacao Nacional* population. Other prospective expeditions were made on the complex *Nacional* × *Trinitario*, in 1942, and in the Ecuadorian Amazon (1949). The collections were established in the Tenguel Farm (Current Centre of Cocoa Aroma Tenguel) and in the Pichilingue Farm. This resulted in the collections of the *refratarios* and silecia.

The first commercial hybrids were released in 1959. And in 1973 a new collection of Amazon cocoa was gathered in the East Ecuador and a new group of commercial hybrids were released in 1975 (Chalmers 1973). From 1983, a group of commercial clones was distributed to the growers for planting. At the same time Allen carried out another expedition in order to collect cocoa types in the East Ecuadorian Amazon. In 1996, a collection of *Cacao Nacional* were established with *arriba* flavour and now the most promising genotypes of that collection are being tested as clone in the multi-location trials. Recently, a total of 108 progenies and 60 clones are under field evaluation. Besides, a new group of crosses is also being initiated involving the Amazon types (Chalmers' selections) and the *Cacao Nacional*.

A participatory approach is being tried with local farmers, with the objective of identifying and selecting high yielding planting material, with resistance to witches' broom, frosty pod rot, and *Phytophthora* pod rot, especially within the population of *Cacao Nacional*. Nestlé promoted this process among the cocoa producers by awarding a prize to those who selected the best planting materials, emphasizing the resistance to witches' broom, yield, yield components and quality.

The Trinidadian Programme – In Trinidad and Tobago two breeding programmes were carried out: one by the Imperial College of Tropical Agriculture (ICTA) and the other by the Ministry of Agriculture (MA). These two programmes established the bases for most programmes undertaken in other countries. In 1930s, Pound characterised the variability existing in the Trinidad's population and, subsequently, selected 100 plants from that elite population. Parallel to the selection of these plants, in the ICTA, a protocol for vegetative propagation of cocoa (rooted cuttings) was developed, by Pike (1933) and Evans (1951), stimulating the test and use of those plants as clones. These plants became the ICS (Imperial College Selection) clones. A series of clonal trials were planted with those clones and lately, some of them were widely planted in Trinidad and distributed among most of the producer countries for breeding varieties. Also, several crosses were made involving these clones, but they did not outperform the parents. In 1937, with the increasing severity of witches' broom disease (*C. perniciosa*) in the country, associated with the low level of resistance in the local population, including the ICS selections, germplasm collecting expeditions (1937/38 and 1942/43) were done in the Amazon region, aiming to collect sources of witches' broom resistance and diversity. In these expeditions, around 2,000 genotypes were collected and planted at Marper station in Trinidad and the two best sources of resistance to witches' broom known up to now, were found – the Scavinas 6 and 12. In these two expeditions, many clones (Parinaris, Pounds, IMCs, Scavinas, etc) widely and intensively used in most cocoa breeding programmes in the world were collected. In 1950s other expeditions (the Anglo-Colombian expedition) were made to the region.

Although the Scavinas were highly resistant to witches' broom they presented an unacceptable seed size and so, could not be released directly to farmers, as it was done with the ICS clones. Crosses were made aiming to combine the good resistance of the Scavinas, with the good seed size and high yield of the ICS clones in the ICTA as also in the MA breeding programme. Unexpectedly, these crosses proved to be very early bearers and highly productive. Therefore, hybrid vigour

was observed for the first time in cocoa. Because of the severity of witches' broom in Trinidad, the breeding efforts were concentrated in developing highly productive resistant varieties. In order to attain these objectives, several strategies of population breeding were practiced, including the development of (a) single crosses involving resistant clones (Sca-6, Sca-12, IMC-67) and heavily bearers (ICS clones); (b) inbred parents to fix some alleles and to generate more uniform hybrids; (c) three-way and more complex crosses, aiming to combine traits spread in more than two parents (e.g., high yield, seed size, seed quality, resistance to diseases, etc); and (d) backcrosses and sib-crosses involving Scavinas. Another important contribution of the ICTA programme was the studies carried out by Cope (1962) to elucidate the mechanisms of sexual incompatibility in cocoa.

The breeding programme of the Ministry of Agriculture was started in early 1930s and its main focus was to combine the accessions with resistance to witches' broom with the ICS selections. In 1949, B.G. Montserin intercrossed the Amazon (IMC-67, Pa-46, Pa-56, Pa-121, Sca-6) selections with the ICS-1. Twenty-one plants were selected out of the 559 available and then backcrossed with both parents. Later, other selections were generated such as the TSH and the TSA (Freeman 1969, 1975) and some of them were distributed to producer countries and are being used for breeding purpose or as planting material.

16.10.2 Asia and Oceania

16.10.2.1 The Papua New Guinea (PNG) Programme

The Cocoa and Coconut Research Institute carried out the cocoa research in PNG. In this country, more emphasis has been given to the development of cocoa hybrid varieties where Upper Amazonian and locally adapted *Trinitario* clones are the main sources as parents. The resulting varieties have been released as planting material to the growers in a mixture of approximately 15 different hybrids. Since 1994, this mixture was modified to include only 10 crosses in two groups (SG2-B and SG2-S) of 5 crosses each based on their potential vigour. A high degree of tree-to-tree variability was observed in yield and a high proportion of unproductive trees. For this reason, the local breeders are giving more attention to the development of clone varieties in order to achieve more uniform plantings with high yielding clones. In order to clone the more adaptive and productive cocoa trees, selections are made within families to be released as hybrid clones. This represents the new production system that is being adopted in PNG since 2003. Appropriate selection of good parents for hybridisation is also essential for an efficient selection programme of new, productive hybrid clones (Efron et al. 2003a).

The hybrid vigour expressed in crosses between unrelated, geographically distinct populations is being used to develop hybrids or hybrid-derived clones. As this characteristic is under genetic control, it is clearly possible to develop clones with desirable size by breeding. The search for uniformity in vigour is important even for clonal varieties, because it reduces the interplant competition and facilitates the

agronomic practices of management and other recommendations. In this manner, clones are being selected according to their vigour to be used in different density of plantings. It has been shown experimentally that the small clones respond better to increased density than the big and medium clones (Efron et al. 2003b,c). Besides that, research is also been carried out with rootstocks in order to explore the interaction between the scions and the rootstocks that favour plantings in higher density. The dwarfing status of the rootstocks was observed to significantly influence the growth of the scions (Efron et al. 2003d). The relationship between yield, vigour and yield efficiency are important in clone evaluation trials planted at one density, but selection of clones based only on yield potential will tend to favour the more vigorous clones.

16.10.2.2 The Malaysian Programme

The Department of Agriculture (DOA) initiated the cocoa breeding programme in the 1930s; with the formation of a germplasm collection constituted by trees selected in the population of *Trinitario* trees, in Serdang, and local *Criollo* materials. Later on, in 1972, the Malaysian Agricultural Research and Development Institute (MARDI) assumed the breeding programme in the Peninsular Malaysia. Some private plantation companies such as Golden Hope Plantations (HMPB) and BAL Plantations also developed some cocoa breeding and selection works. A large number of high yield planting materials were developed and released to the producers at that time. In 1989, a new agency, the Malaysian Cocoa Board (MCB) was created for co-coordinating and integrating the activities of the cocoa industry that was experiencing a rapid expansion.

The cocoa breeding programme that has been carried out by the MCB gave emphasis to improving plant materials for yield, bean quality, pest tolerance, disease resistance and for traits that could facilitate field management operations. The vascular streak dieback, caused by *Oncobasdium theobromae* is one of the most serious cocoa diseases in Malaysia, followed by *Phytophthora* pod rot. And the cocoa pod borer is the most important pest that causes large pod losses. Work was carried out in two lines of breeding: hybrids and clones. According to Ooi et al. (1989) about 118 hybrid trials, involving 2,206 progenies have already been evaluated in Malaysia. These hybrid trials represent three series. The first hybrids recommended to producers involved *Amelonado* parents, but later on, other hybrids, such as *Trinitario* × Amazonian (UIT1 × NA33) and Amazonian × Amazonian (Pa7 × Na33) and others of the series II and III are expected to produce over 2,500 kg/ha/yr and most of them, specially the ones of the series III have shown tolerance to vascular streak dieback. The development of clones was initiated in 1956, by the DOA by selecting outstanding cocoa trees within and between progenies. Now, governmental and private research organisations are participating together in this programme. A large number of clones have already been evaluated in various clonal trials, and most of them are being tested under different environmental conditions. About 30 high yielding clones have been recommended as planting material and some of them also exhibit high degree of tolerance to VSD, as for example PBC-123, BAL-209 and

KKM-25 (Chong et al. 1989). They are more concerned in improving varieties for high yield and that present more local adaptation. For this, single crosses, double crosses, three-way crosses and recurrent crosses are used to produce the progenies. Many of these crosses are produced according to a genetic mating design, allowing, inclusive, the estimation of genetic parameters, such as the combining ability.

16.10.3 Africa

The Lower-Amazon *Forastero* cocoa types were introduced for the first time in 1857 to the São Tomé Island and after which into the African Continent (Burle 1952). The cocoa plantations were intensified in the beginning of the 19th Century by the European colonisers with their influence in West and Central Africa. In West Africa, the first plantations were established mainly from Lower-Amazon *Forasteros*, called more commonly *Amelonado*. These cocoa trees have a relatively narrow genetic base. In Central Africa, *Trinitario* and Lower-Amazon *Forastero*, constituted the first plantations of the region. The cocoa production comes essentially from small-holdings. Today, Ivory Coast and Ghana, with respectively, 1,300,000 tons and 600,000 tons, produce 60% of the world cocoa production.

Ivory Coast breeding programme – The *Centre National de la Recherche Agronomique de Ivory Coast* (CNRA) has based its selection strategy on the hybrid varieties. These hybrids come from crossings between Lower-Amazon *Forasteros* (*Amelonado* type) and Upper-Amazon *Forasteros* or *Trinitarios*. Hybrids between, Upper-Amazon *Forasteros* and Lower-Amazon *Forasteros*, present more elevated production levels that the original cocoa trees (*Amelonado* type). Sometimes, the *Trinitario* parents have been preferred to the Lower-Amazon *Forasteros*, in order to better merge yield and bean size at the same time, as an example, hybrids with UF-676 or UF-667. However, the heterozygosity is often important within these hybrid progenies. The level of production of the first generations of hybrids that were distributed to the farmers in the 1970s, yielded approximately two tons of dry beans per, twice as much as Lower-Amazon *Forasteros* (*Amelonado* type). The productions of the hybrid combinations between Upper-Amazon *Forasteros* and *Trinitarios* are lower than those observed for the hybrids between Upper-Amazon *Forasteros* and Lower-Amazon *Forasteros*. A second hybrid programme permitted an increase the productivity with the introduction of new progenitors of the Upper-Amazon *Forastero* population (Paulin et al. 1994; Clément et al. 1999). This second cycle of hybrid varieties led to gain in production of 30–70% in relation to the first generations of hybrids, comparatively.

The idea in the use of doubled haploids (DH) for developing cocoa hybrid varieties was also put in practice in Ivory Coast. (Sounigo et al. 1994, 2003). Doubled haploids was produced by colchicine treatment of either spontaneous haploid cocoa seedlings or induced through pollen radiation (Falque 1994). As the frequency of spontaneous haploids was very low, large progenies needed to be produced, and the occurrence of haploids varies among different progenies, that caused the process to

be fairly costly. The same difficulty was found using the induction process. Besides, this line of investigation had no continuity.

Breeding hybrid varieties by crossing parents from different geographical groups did not permit to achieve the aim of a continuous genetic progress. The previous population improvements for the development of hybrids have been considered, in order to increase the frequency of favorable alleles in the populations. The goal of using successive cycles of recurrent selection has been proposed in 1988, and a first cycle was produced in 1990 (Eskes et al. 1995). The scheme is based on recurrent selection established from two populations: the first one, represented by crossings between Lower-Amazon Forestero genotypes and some *Trinitario* genotypes (African *Trinitario*), and the second one, represented by crossings between Upper-Amazon *Forasteros*. In the populations, parents have been selected with respect to their individual genotypic value as well as to their general combining ability, while taking into account available information on the genetic structure of cocoa groups, obtained mainly with isozyme markers (Lanaud 1986). This recurrent selection scheme foresaw the achievement of an intra-populational improvement in two cycles, and then, the second-cycle progenies would engage in crossing between selected genotypes from these two populations for the development of new varieties. This strategy foresees the possibility for selecting clones or hybrids in every cycle of recurrent selection. The choice of genotypes to produce the second cycle has been made on the genetic progress with respect to yield, vigor and reaction to *Phytophthora* pod rot through inoculation leaf tests (Lachenaud 2000).

Various hybrids present important genetic variability for resistance to the *Phytophthora* pod rot. In Cameroon, resistant clones to *Phytophthora megakarya* have been selected with the aid of inoculation leaf tests (Nyassé et al. 1995). In Ivory Coast, clones and hybrids (Tahi et al. 2000) have been identified as resistant to *Phytophthora palmivora* through field evaluations. Index selection has also permitted the selection of some remarkable trees among and within hybrid progenies in respect to *Phytophthora* pod rot resistance and yield, (Cilas et al. 1999a). Results from different countries as Cameroon, Togo and Ivory Coast showed that some the clones presented an equivalent resistance level in all these locations (Cilas et al. 1999b). A large project, for selecting resistant clones or progenies to *Phytophthora* spp disease has been carried out by Ivory Coast, Cameroon, Trinidad and Cirad. The European cocoa manufacturer association (CAOBISCO) has financed this project. The main results have been published in a book entitled: Improvement of cocoa tree resistance to *Phytophthora* diseases. (Cilas and Desprèaux 2004).

During the last ten years, studies on genetic mapping and QTL detection have been carried out in segregant populations of the Ivory Coast breeding programme. The genetic reference map of *Theobroma cacao* L. has been developed using the progeny UPA402 × UF676 (Lanaud et al. 1995; Pugh et al. 2004). And, QTLs for resistance to *Phytophthora* also, for agronomic and quality traits were identified (Lanaud et al. 1999; Flament et al. 2001; Risterucci et al. 2003; Clément et al. 2003a,b).

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